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POSSIBLE INVOLVEMENT OF HYPOTHALAMIC ADRENALINE  
IN THE SEXUALLY DIFFERENTIATED RESPONSE OF LH TO AN  
OESTROGEN STIMULUS IN IMMATURE RATS

by

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FEBRUARY, 1986

*Date of submission: February 1986*

*Date of award: 11 June 1986*

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DEDICATED TO MY BROTHER

This thesis is dedicated to the memory of my brother, P. Clement,  
who died in November, 1972, at 12 years of age.

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## ACKNOWLEDGEMENTS

I have great pleasure in expressing my deep and sincere gratitude to my supervisors, Dr. S. Murphy of the Open University for his encouragement and, in particular to Dr. P. C. B. MacKinnon of the Oxford University for her support and guidance during the course of my research in her laboratory. I am also grateful to Professor R. Guillery FRS, Dr. Lee's Professor of Anatomy for his interest in my project. This work would certainly have been difficult had I not taken kind help and advice of Dr. Richard Sheaves and Mr. Robert Laynes.

I am indebted to Professor G. Niswender (Department of Physiology and Biophysics, Colorado State University, Colorado 80523) for an antiovine LH serum No. 15, to Professor L. Reichert (Department of Biochemistry, Albany Medical College, New York 12208) for purified ovine LH for iodination; to the National Institute of Arthritis, Diabetes, Digestive Diseases and Kidney (NIADDK) for LH standards (NIH-LH-S13), to Burroughs Wellcome for anti-rabbit gamma globulin (ARGG) and to the Wellcome Trust for providing financial support during the course of my research work (Grant No. 13181/1.5/DG/LA/S1p to Dr. MacKinnon).

Last, but not least, I thank my wife, Shima, and children, Ambreen and Amar, who have shared with me the best and worst of research life.

### DECLARATION OF PUBLICATION

Most of the work reported in this thesis has recently been accepted by the Journal of Endocrinology for publication.

## ABSTRACT

In the adult rats an increase in adrenergic activity in the medial preoptic area (MPOA) and the mediobasal hypothalamus (MBH) occurs during the proestrous 'critical period' preceding the preovulatory gonadotrophin (LH) surge. Similarly, using adult rats ovariectomized in the morning of dioestrus and injected (s.c.) immediately with oestrogen ( $OE_2$ ) it was found that adrenergic activity increased in the MBH but not the MPOA during the expected 'critical period'. It was suggested from these observations that an oestrogen stimulated increase in adrenergic activity in the MBH might be concerned with the surge output of gonadotrophin. In a previously reported ontological study of an oestrogen stimulated output of gonadotrophin, it was found that the occurrence of this neurohumoral event was critically related to the age and sex of the animal. An injection (s.c.) of oestrogen at noon (day 1) in infant rats at as early an age as 21 days could elicit a surge of prolactin at 1800h the next day (day 2) and again on day 3; while a surge of LH occurred only at 1800h on day 3. Similar treatment of males at any age had no such effect. The purpose of the studies presented in this thesis was to investigate the development of central adrenergic systems concerned with the ontogeny and sexually differentiated output of gonadotrophin in response to an oestrogen stimulus in the infant rats.

Adrenaline concentration and the activity of its synthesizing enzyme (phenylethanolamine-N-methyl transferase, PNMT) present in the MPOA and MBH of oestrogen treated 16-, 22-day-old females and 22-day-old males were measured. This showed that adrenaline concentration and activity of PNMT in the MBH of oestrogen treated 22-day-old female rats increase

significantly ( $P < 0.01$ ) at 1600h both on days 2 and 3, 2h before the oestrogen dependent surge output of gonadotrophins (i.e. prolactin with or without LH) reach peak levels (1800h). Such an effect was not seen in similarly treated 16-day-old female and in 22-day-old male rats on day 3, with the exception of PNMT activity in 22-day-old males which showed an increase similar in the female. 22-day-old oil-treated male rats also showed an increase in PNMT in the MBH at 1600h which was not significantly different from those of oestrogen-treated rats. Noradrenaline and dopamine concentrations in the MBH of oestrogen treated 22-day-old female rats remained at baseline levels on days 2 and 3. In contrast to the MBH, the MPOA of these rats did not show an increase in either adrenaline concentration or PNMT activity on day 3. Subsequent measurements of PNMT activity in the MBH of oestrogen treated 22-day-old female rats at 4 hourly intervals throughout the days 2 and 3 showed the presence of a clear circadian rhythm with peak levels occurring at 1600h. This rhythm appears to be dependant on the presence of plasma oestrogen and glucocorticoids since oil-treated or adrenalectomized 22-day-old females did not show an increase in PNMT at 1600h.

In conclusion, it is clear that an increase in adrenaline concentration in the MBH of oestrogen treated 22-day-old female rats appears to be in synchrony with an increase in its synthesizing enzyme, PNMT. Furthermore, it is temporally related to surge output of gonadotrophins (LH and prolactin) which occur 2h later (1800h). Whether or not the temporal relationship occurring in the oestrogen-treated 22-day-old females is directly concerned with the output of gonadotrophins, or with other hormones which increase at the same time, is a question which requires investigation.



GENERAL INTRODUCTION

The preovulatory surge of gonadotrophins - luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the rat is accompanied by a surge of a decapeptide known as gonadotrophin - releasing hormone (GnRH) (Sarkar et al., 1976; Schally, 1978; Guillemin, 1978). This decapeptide is released into hypothalamo-hypophysial portal vessels which connect the base of brain, in particular the median eminence (ME) with the anterior pituitary gland which is devoid of connecting neural pathways. Observations on the rat (Green & Harris, 1949) and mouse (Worthington, 1955) have shown that the direction of blood flow in the portal vessels is from the brain towards the gland. However, it has been observed recently that flow in the reverse direction can occur in part of the plexus of portal vessels and therefore, pituitary hormones may reach the hypothalamus (Page et al., 1976; Oliver et al., 1977). This later observation may account for the mechanism by which the pituitary regulates its own secretions (short-loop feedback). Using immunocytochemical techniques, it has been demonstrated that GnRH cell bodies in the rat are localized in and around the medial preoptic area and that their axon terminals project largely to the lateral aspects of the outer layer of the median eminence (Kordon et al., 1974; Baker et al., 1975; King et al., 1982).

It is well established that the preovulatory surge of gonadotrophins accompanied by a surge of GnRH is not only preceded by but is also dependent on increased output of plasma oestrogen (Legan et al., 1975; Fink, 1976) and furthermore, by a neural critical period, (Everett & Sawyer, 1950; Legan & Karsch, 1975). This critical period

can be defined as about a 2h period on the proestrous afternoon during which if phenobarbitone is administered in anesthetic dose blocks the proestrous preovulatory LH surge.

Since previous studies have indicated that hypothalamic adrenaline might play a critical role in the output of LH surge during the neural critical period in adult cyclic rats (Mackinnon et al., 1983, 1985), the present studies were undertaken to investigate the ontogeny of hypothalamic adrenaline in the oestrogen-stimulated LH surge and secondly, its possible sexual differentiation.

#### Preovulatory increase of plasma oestrogen:

Follicles form the basic functional unit of the rat ovary and are responsible for the increased plasma oestrogen. Immature follicles known as primordial follicles grow initially under the local ovarian control forming primary follicles. Each primary follicle consists of an outer layer of theca interna cells which encircles the granulosa cells and a basement membrane separating internal theca from those of the granulosa. Granulosa cells also surround the innermost oocyte cumulus complex. The whole follicle is enclosed in a layer known as the theca externa which contains contractile elements. Further maturation of the primary follicles to antral or preovulatory follicles and their transformation into corpora lutea is regulated by a set of pituitary hormones namely FSH, LH and to a lesser extent prolactin which plays a supportive role (Richards, 1978; Henderson, 1979).

#### (a) Hormonal regulation of ovarian follicular development:

The length of the rat ovarian cycle (the oestrous cycle) lasts in a

majority of animals for 4 days and consists of proestrous, oestrous, metoestrous and dioestrous stages. Cyclic release of pituitary gonadotrophins interacts with ovarian follicular compartments in a highly integrated manner and is responsible for their development. As development proceeds follicular fluid is retained within the growing follicle. FSH and oestrogen are found in this fluid which fulfils the essential requirement for further growth of the follicles (Richards, 1978).

During the early oestrous period, FSH is the prime inducer of ovarian follicular maturation (Chappel et al., 1983). However both FSH and LH are required for oestrogen biosynthesis. One piece of evidence for this came from a study of immature rats in which they were hypophysectomized at 28 days of age and 7 days later injected (s.c.) with ovine interstitial cell stimulating hormone (ICSH) and ovine FSH (Lostroh & Johnson, 1966). This study demonstrated that both ICSH and FSH were essential for follicular development and oestrogen production. It is widely accepted that the increased production of oestrogen from a preovulatory follicle when growing under the control of gonadotrophins is due to the co-operation of two types of ovarian cell, the theca interna and the granulosa. This so called two cell and two gonadotrophins concept (Fig.1) (Dorrington et al., 1977) is a useful model for understanding the biosynthesis of oestrogen in ovarian follicles. This concept is supported by a large number of both in vivo and in vitro studies. An in vivo, classical study by Falck (1959) in which both theca interna and granulosa cells were transplanted to the anterior chamber of the rat eye, showed that the joint action of two cell types was necessary for the biosynthesis of oestrogen. An autoradiographic study using rat ovarian tissue

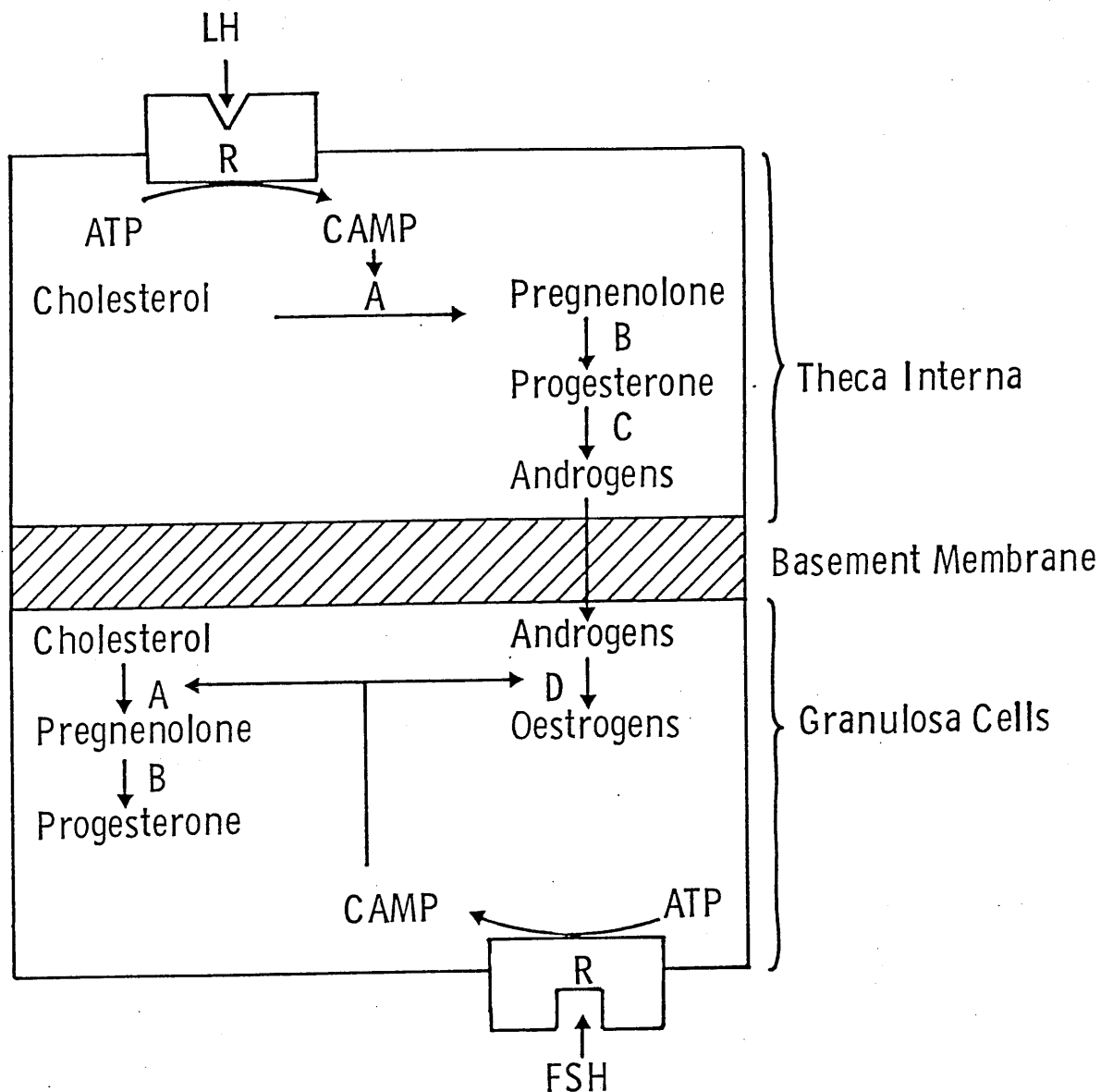


Fig. 1. Simplified diagrammatic representation of the two cell, two gonadotrophins concept

- A. C - 20, 22 - side chain cleavage enzymes
- B.  $3\beta$  - hydroxy steroid dehydrogenase & isomerase
- C. C - 17, 20 - side chain cleavage enzymes and  $17\beta$  - hydroxy steroid dehydrogenase
- D. Aromatizing enzymes
- R. Receptor

demonstrated that radioactive LH was bound to theca interna cells whereas granulosa cells were devoid of such binding (Richards & Midgley, 1976). In vitro studies of isolated theca interna cells have demonstrated that these cells secrete predominantly androgens [androstenedione and testosterone] in response to LH stimulation and have relatively little capacity to metabolise androgens to oestrogen (Fortune & Armstrong, 1977; Ahren et al., 1978). This is because of the low activity of the aromatizing enzymes, catalyzing; (a) the reduction of keto to hydroxyl group ( $3\beta$ -hydroxy steroid dehydrogenase), (b) the cleavage of C10-C19 bond (C-10,19-side Chain cleavage enzyme), (c) the introduction of double bonds which lead to the formation of oestrogen. In contrast to LH, FSH binds exclusively to the granulosa cells, however their binding capacity for LH is developed during late proestrous stage due to newly synthesized LH receptors on these cells which are induced by FSH and oestrogen (Richards & Midgley, 1976; Richards, 1979). In vitro studies have shown that granulosa cells lack the enzymes, C17, 20 - side chain cleavage enzymes and  $17\beta$ -hydroxysteroid dehydrogenase which convert progesterone (C21-compounds) to androgens (C19-compounds) (Dorrington, 1977; Channing et al., 1980; Hillier, 1981). However, granulosa cells are capable of converting cholesterol to progesterone due to the presence of enzymes, C-20, 22-side chain cleavage enzymes,  $3\beta$ -hydroxy steroid dehydrogenase and isomerase which metabolise cholesterol to progesterone. Furthermore the granulosa cells can metabolise oestrogen when provided with androgens (Dorrington et al., 1975) due to the presence of enzymes which catalyze aromatization (Kase & Speroff, 1980). According to the two cell, two gonadotrophins concept, FSH activates the aromatizing enzymes and furthermore, stimulates progesterone biosynthesis in the granulosa cells. LH interacts with the theca interna through its

specific receptors and stimulates biosynthesis of androgens from cholesterol. Androgens diffuse through the basement membrane into the granulosa cells and are converted to oestrogen. Oestrogen with FSH, in turn, induces more LH receptors on theca interna cells and consequently an enhanced response to LH. This causes these cells to produce more androgens which diffuse into granulosa cells and consequently a much higher output of oestrogen. This intra-ovarian positive feedback effect of oestrogen is primarily considered to be responsible for the preovulatory rise of oestrogen which occurs during late dioestrus and early proestrus.

(b) Negative feedback:

The rising titers of oestrogen on late dioestrous initially induce a longloop negative feedback effect on gonadotrophins - LH and FSH (Martini et al., 1968a,b; McCann & Ramirez, 1964). Current radio-immunoassays are probably not sensitive enough to detect small fluctuations in plasma gonadotrophin concentrations in intact cyclic rats, except on the proestrous day. Therefore evidence for negative feedback is derived, largely from studies on ovariectomized rats. The removal of rat ovaries leads to increased pituitary and plasma gonadotrophin levels, and was first described by Moore & Price (1932). Furthermore, it was found that the administration of exogenous oestrogen to the ovariectomized rats caused LH levels to decrease.

More recently, in confirmation of previous studies, an investigation involving long-term ovariectomized rats with indwelling atrial cannulae demonstrated that subcutaneous administration of a heavy dose of 50mg oestradiol benzoate depressed the enhanced post-ovariectomy plasma LH concentrations which occurs in a pulsatile manner (Blake, 1977). The complex series of neural events involved in negative feedback are

not fully understood. It is generally accepted, however, that the mechanisms underlying the post-ovariectomy pulsatile release of LH lies within the mediobasal hypothalamus (MBH) and, furthermore, its interaction with the anterior pituitary is essential for mediating the feedback. Evidence for this comes largely from three different types of investigation. Studies pertaining to the interaction between the pituitary and brain suggest that pituitary LH release is modulated by GnRH output since portal plasma GnRH concentrations are increased in ovariectomized rats (Sarkar & Fink, 1980; Sherwood & Fink, 1980). In confirmation of this work, an in vitro study showed that anterior pituitary fragments from ovariectomized rats released LH in a pulsatile manner only when driven by the infusion of pulsatile GnRH in the medium (Osland et al., 1975). In another investigation, steel cannulae containing oestradiol were implanted stereotaxically into the mediobasal hypothalamus (MBH), anterior pituitary, globus pallidus and supra-chiasmatic region of ovariectomized rats (Ramirez et al., 1964). The data showed that implants of the anterior pituitary or the MBH prevented the post-ovariectomy rise in LH concentration whereas implantation into other regions showed no such effect. This study clearly indicated that the MBH and anterior pituitary were the site of negative feedback. In the third type of study using female rats, surgical cuts were made stereotaxically either between the medial preoptic area (MPOA) and the MBH or a complete deafferentation of the MBH was undertaken. These animals were ovariectomized and then implanted with indwelling atrial cannulae for the collection of serial blood samples (Blake & Sawyer, 1974). This study demonstrated that the MPOA-MBH axis and complete-deafferentation of the MBH did not reduce the post-ovariectomy rise in LH when compared to non-deafferentated ovariectomized rats. Furthermore, this study implied that MPOA was not the site

the negative feedback since the enhanced post-ovariectomy LH levels were found even after surgical cuts were made between the MPOA and MBH.

The subcellular effects of oestrogen in mediating the negative feedback are probably achieved through genomic mechanisms. Autoradiographic studies have shown that nuclei of pituitary gonadotrophs concentrated radioactive oestradiol (Stumpf, 1971). Moreover, the removal of the negative feedback effect of oestrogen changed the pituitary sensitivity by increasing the density of GnRH receptors (Drouin et al., 1976; Drouin & Labrie, 1981). The post-ovariectomy rise in LH concentration is probably due to an enhanced responsiveness of the anterior pituitary to GnRH. Recently, the possibility of a direct effect of oestrogen on plasma membrane has gained favour as oestrogen appears to alter ionic channels (Kelly, 1982). An electrophysiological study on guinea pig MBH demonstrated some immediate alterations in the electrical activity in response to oestrogen (Kelly & Ronnekleiv, 1982). However, a similar demonstration of GnRH neurones responding to oestrogen in the rat MBH has not been reported in the literature. Such a study might provide new dimensions in our understanding of feedback of oestrogen in LH release.

(c) Positive feedback:

It is generally accepted that the preovulatory surge in outputs of pituitary hormones, prolactin (Frantz et al., 1972; Kalra et al., 1973; Neil, 1974; Leong et al., 1983), corticotropin (ACTH, Buckingham et al., 1978) and thyrotrophin (TSH, Brown-Grant et al., 1977; Buckingham et al., 1978) is preceded by enhanced plasma oestrogen levels originating from the ovarian follicles. These enhanced levels of oestrogen



which are achieved for a specific duration of time (both parameters being species specific) between the dioestrous and proestrous stage, elicit a long-loop positive feedback on gonadotrophins - LH and FSH (Fink, 1979; Chappel et al., 1983).

It is well established that an increase in the concentration of plasma oestrogen is an essential requirement for the preovulatory surge of LH release which causes ovulation (Brown-Grant et al., 1970; Fink, 1976; Chappel et al., 1983). Evidence to support this comes from a study by Ferin et al. (1969) in which it was shown that antisera, which blocked the biological activity of oestradiol-17 $\beta$ , when administered (i.p.) to immature rats treated with pregnant mare serum, also blocked ovulation. Furthermore, in these 'blocked' rats, diethylstilboestrol (a synthetic oestrogen which does not bind to the antisera) restored ovulation. Further evidence was derived from studies with rats ovariectomized in the morning of dioestrus and injected (s.c.) immediately with oestradiol benzoate (OE<sub>2</sub>) (acute model). This caused a proestrous-like surge the following evening while the control group of ovariectomized rats injected with oil vehicle showed no such effect (Tapper et al., 1974). In another study rats were ovariectomized and 5 to 8 weeks later injected (s.c.) with OE<sub>2</sub> (chronic model). This steroid stimulus caused a proestrous-like LH surge 78h after the initial injection (Aiyer et al., 1976). The mechanism by which oestrogen induces a positive feedback response of LH is not fully understood. However, it is generally accepted that oestrogen brings this about by its effects at both pituitary and hypothalamic levels. Using an oestrogen exchange assay in which rats were ovariectomized and injected (i.v.) with saturating levels of oestrogen, oestrogen binding to its receptors was measured (Clark et al., 1982). This study demonstrated that oestrogen

binding occurred not only at the pituitary level but in discrete areas of the hypothalamus (MPOA and MBH) and the brain stem.

Two phenomena have emerged in order to describe the oestrogen response at the pituitary;

(a) Pituitary responsiveness:

this is described as its capability to release LH and FSH in response to GnRH. Pituitary responsiveness increases between the afternoon of dioestrous and that of proestrous whereas it is negligible during other days of the oestrous cycle. Using intact regularly cyclic rats administration of GnRH through the external jugular vein showed that LH in the peripheral plasma was significantly higher during the proestrous afternoon as compared to other days of the cycle (Aiyer et al., 1974a). Similarly, in another study using acutely ovariectomized rats, it was demonstrated that the enhanced sensitivity of the pituitary to GnRH during proestrus depends on the increase in plasma oestrogen which precedes the preovulatory surge of LH, no effect having been seen in ovariectomized rats (Aiyer & Fink, 1974). Furthermore, administration of an antioestrogen (ICI46474) on dioestrus reduced the LH response to GnRH injected (i.v.) on the afternoon of proestrous which implies that the essential role of oestrogen in enhancing the pituitary responsiveness to GnRH is indispensable. These in vivo results are supported by an in vitro study using pituitary fragments which clearly indicated that the proestrous pituitary was much more sensitive to GnRH as compared to pituitary fragments taken on other days of the cycle (Waring & Turgeon, 1980). The mechanism by which oestrogen increases pituitary responsiveness is probably through genomic mechanisms by enhancing the number but not the affinity of GnRH receptors. The number of pituitary GnRH receptors parallels the magnitude of the LH

output in response to GnRH (Ferland et al., 1981); and it has been demonstrated that the number of GnRH receptors varies markedly during the oestrous cycle in rats, more receptors being found during proestrus than at any other time of the cycle (Savoy-Moore et al., 1980; reviewed by Clayton & Catt, 1981).

(b) Self-priming effect:

The preovulatory output of LH and FSH is dependent not only on oestrogen sensitization of the pituitary, but also on a self-priming effect of GnRH. This is described as the oestrogen-primed gonadotrophs ability to release a greater amount of both LH and FSH in response to a second bolus of GnRH as compared to the first. Thus an investigation using three groups of rats (intact cyclic, acutely ovariectomized injected with oil vehicle and acutely ovariectomized injected with OE<sub>2</sub>) demonstrated that the proestrous output of LH into peripheral plasma was much greater in response to the second bolus of GnRH injected (i.v.) one hour after, as compared to oestrus, metoestrus and dioestrus stage of the cycle (Aiyer et al., 1974b). The ovariectomized-OE<sub>2</sub> treated animals showed that the self-priming effect of GnRH was similar to that obtained in proestrous animals but the ovariectomized-oil treated were devoid of such effect. The FSH response to the second bolus of GnRH, given one hour after the first, was found to be too small to be significantly different from that of the first FSH response. Using proestrous anterior pituitary fragments in a perfusion system, Waring and Turgeon (1980) have demonstrated the 'self-priming' effect of GnRH in releasing LH which was similar to that in vivo.

Intra-cellular mechanisms involved with GnRH self-priming are still not fully known. However, the synthesis of new protein and functional integrity of microfilaments within the gonadotroph have been shown

to be an essential part of the mechanism. Evidence for synthesis of new protein came from an investigation by Pickering and Fink (1976) in which inhibitors of protein synthesis (cycloheximide, puromycin and actinomycin) were either injected (i.v.) into proestrous rats or introduced to medium perfusing fragments of anterior pituitary from proestrous animals. This in vitro investigation demonstrated that the inhibitors had little effect on the first exposure to GnRH. However, a second exposure to GnRH significantly reduced LH output. It was thought that the synthesis of new protein caused the formation of new LH molecules during the self-priming of GnRH, but the assessment of measurement both of gonadotrophins released and in the pituitary did not support this hypothesis (Pickering & Fink, 1979, Speight & Fink, 1981). In a recent study using two-dimensional gel electrophoresis, newly synthesized protein, present in the hemipituitary exposed to GnRH, was extracted and characterized (Curtis et al, 1985). This study showed that GnRH priming was associated with the synthesis of a new high molecular weight protein and post-translational changes in two previously existing proteins; the synthesis of new LH, however, was not involved. The dependence of GnRH-priming on cellular microfilaments was investigated by Pickering & Fink (1979). This investigation showed that cytochalasin B (a potent inhibitor of the functional integrity of microfilaments) when infused into the medium perfusing hemipituitaries, inhibited the enhanced output of LH in response to a second bolus of GnRH.

The role of microfilaments in the self-priming effect of GnRH has been further studied using proestrous mice hemipituitaries perfused for two consecutive hours with or without cytochalasin B (Lewis et al., 1985). In this study, the pituitary gonadotrophs were identified using immunocytochemical techniques and the ultrastructural changes in

gonadotrophs exposed to GnRH were characterized by electron microscopy. This study, a confirmation of the previous investigation of Pickering & Fink (1979) demonstrated that the output of LH in response to the first bolus of GnRH was unaffected by cytochalasin B, but the enhanced output of LH in response to the second bolus was abolished. Moreover, with respect to the ultrastructural correlates, it was shown that the exposure of proestrous anterior pituitary in vitro to the first bolus of GnRH resulted in the formation of smaller secretory granules and their migration into the marginal zone beneath the plasma membrane. This was also associated with longer microfilaments and changes in their orientation, in that they came to lie in close proximity to the plasma membrane. The injection of a second bolus of GnRH increased further the migration of smaller secretory granules towards the periphery of the plasma membrane and caused changes in orientation of the microfilaments. Moreover, a significant drop in the secretory granules was observed after the second bolus. The functional integrity of microfilaments and processes leading to the migration of smaller secretory granules towards the periphery of the plasma membrane during GnRH self-priming, is clearly associated with the output of a readily releasable pool of gonadotrophins.

#### The neural 'critical period'

It is well accepted that the positive feedback response to oestrogen is mediated at the MPOA-MBH unit which is involved in the synthesis and output of gonadotrophin releasing hormone. Furthermore, the response is also dependent on the establishment of a neural 'critical period' (reviewed by Fink, 1979; and Kalra & Kalra, 1983). Indications that oestrogen mediates its effects at the MPOA and MBH came as a result of autoradiographic studies in which it was demonstrated that [<sup>3</sup>H]-

oestradiol was concentrated at these discrete areas of the hypothalamus (Pfaff, 1968; Stumpf, 1968; Pfaff & Keiner, 1973). However, electro-stimulation of the MPOA using acutely ovariectomized rats injected (s.c.) with oestradiol benzoate (OE<sub>2</sub>) showed a facilitation of GnRH output into the hypothalamo-hypophysial portal vessels (Sherwood et al., 1976). This facilitatory response of OE<sub>2</sub> was, however, ineffective when the MBH was electrically stimulated. Similarly, another study showed that an acute horizontal section placed stereotaxically at the level of the anterior commissure just above the MPOA of cyclic rats, prevented ovulation (Kawakami & Terasawa, 1972). Furthermore, stainless steel cannulae filled with oestradiol when implanted into the MPOA of actually ovariectomized rats with their hypothalamic connections intact, evoked the LH surge, yet similar stimulation of the MBH was devoid of such an effect (Goodman, 1978). Previous classical concepts pertaining to the stimulatory effect of oestrogen at the MPOA have implied that its action is mediated through genomic mechanisms (Pfaff & Keiner, 1973; King et al., 1982). However, a combined autoradiographic/immunocytochemical technique has failed to demonstrate the incorporation of [<sup>3</sup>H]-oestradiol into the nuclei of GnRH cell bodies; instead the tritiated steroid was taken up by small neurones lying in close proximity to GnRH containing neurones (Shivers et al., 1983). This implies that mechanisms involved with oestrogen stimulation other than genomic activation, may be operative at GnRH synthesizing cell body level.

The establishment of the neural 'critical period' in response to oestrogen occurs in the afternoon of proestrus in cyclic rats. This is defined as an interval on the afternoon of proestrus during which the administration of central nervous blocking agents, prevented ovulation the following day (reviewed by Everett, 1964). Evidence for a neural 'critical

period' is derived from the observation that pentobarbital anaesthesia administered during a limited period of the proestrous afternoon (1400-1600h) delayed the occurrence of the LH surge and ovulation by 24h (Everett & Sawyer, 1950), whereas injection (i.p.) of pentobarbital either earlier or later than the critical period, had little or no effect on the preovulatory LH surge or ovulation. In our colony, rats maintained under a controlled lighting regime (lights on: 0600-2000h) have a critical period between 1430-1700h, while the LH surge occurs between 1700-1900h and ovulation between 0200-0400h of oestrus (Sarkar et al., 1976). Pentobarbital does not seem to diminish or abolish the anterior pituitary response to exogenous GnRH and therefore it is likely that it affects the neurogenic mechanisms of the brain involved in the release of GnRH (reviewed by Barraclough & Wise, 1982; and Barraclough et al., 1984). Neural mechanisms underlying the critical period are still unclear, but it is likely that both the MPOA and MBH are involved. Several reports have demonstrated that prostaglandins, opioids and neurotransmitters, in particular catecholamines, are involved with the critical period (described above) which precedes the  $OE_2$ -stimulated LH surge and which is presumably associated with the release of GnRH into the hypothalamo-hypophysial portal vessels (reviewed by Ojeda et al., 1981; Barraclough & Wise, 1982; and Kalra & Kalra, 1983).

a. The role of prostaglandins in the output of GnRH:

It is generally accepted that prostaglandin  $E_2$  ( $PGE_2$ ) is involved in the output of GnRH (presumably during the 'critical period') by acting on the MBH, in particular the ME (reviewed by Ojeda et al., 1981). Evidence to suggest that  $PGE_2$  may play a role in the output of GnRH came from a study in which the blockade of LH output as a result of stereotaxic infusion of either adrenergic, dopaminergic, serotonergic or cholinergic receptor blockers into the IIIrd ventricle of long-term ovariectomized

rats was not only reinstated but also enhanced by the infusion of  $\text{PGE}_2$  into the third ventricle (Harms et al., 1976). This probably implies that  $\text{PGE}_2$  acts directly on GnRH neurones in order to release GnRH into the hypothalamo-hypophyseal portal vessels and hence LH output. Similarly, another study showed that blockade of prostaglandin synthesis with indomethacin prevented the oestrogen-progesterone induced output of LH in long-term ovariectomized rats when indomethacin implants were placed in the MBH before the occurrence of the LH surge (Ojeda et al., 1975). The mechanisms of synthesis of prostaglandin  $\text{E}_2$  and its role in eliciting GnRH output has recently been delineated (reviewed by Negro-Villar et al., 1980; and Ojeda et al., 1981). Unlike most other hormones it probably acts as a local hormone which is synthesized within the MBH under the influence of neurotransmitters, in particular, catecholamines (Ojeda et al., 1979). In this study by Ojeda et al, using MBH and ME fragments of the rat brain in a perfusion system, it was demonstrated that the infusion of NE into the medium was more effective than DA in releasing  $\text{PGE}_2$  concomitant to the GnRH output. The operative role of  $\text{PGE}_2$  on GnRH output is probably through the mobilisation of intracellular and extracellular calcium ions (Ojeda & Negro-Villar, 1984). However, a significant portion of the  $\text{PGE}_2$  effect on GnRH release is independent of extracellular  $\text{Ca}^{2+}$ , since the complete omission of  $\text{Ca}^{2+}$  in the medium perfusing the ME did not prevent GnRH release in response to the depolarization of ME with  $56\text{mM K}^+$  (Ojeda & Negro-Villar, 1985). Furthermore, in this study depletion of intracellular  $\text{Ca}^{2+}$  stores by incubation with  $\text{Ca}^{2+}$  ionophore, A23187 in a medium without  $\text{Ca}^{2+}$  and containing EGTA, almost completely blocked the subsequent GnRH response to  $\text{PGE}_2$ . This suggests that intracellular  $\text{Ca}^{2+}$  stores are essential in mediating the  $\text{PGE}_2$  effect on GnRH output.



b. The role of opioids in the output of GnRH:

Several studies have demonstrated that endogenous opioids induce inhibitory effects on the output of GnRH. It is possible that the removal of this inhibitory effect during the 'critical period' might disinhibit a preovulatory GnRH surge (as reviewed by Kalra, 1982; and Kalra & Kalra, 1983). Thus the administration (i.p.) of morphine (an opiate agonist) to proestrous rats prior to the 'critical period' blocked ovulation the following day (Barraclough & Sawyer, 1955). Similarly, in other studies the administration of morphine to intact or ovariectomized steroid-primed rats blocked the LH surge (Pang et al., 1977; Ieiri et al., 1980; Kalra & Simpkins, 1981). Evidence to suggest that the inhibitory effect of morphine occurs at the level of opiate receptors came from investigations in which it was demonstrated that a specific opiate receptor antagonist, naloxone, stimulated LH release in intact and steroid-primed rats (Meites et al., 1979; Kalra et al., 1981). Presumably the inhibitory influence of opioid peptide neurones on GnRH neurones was prevented. The mode of action of endogenous opioids in the regulation of GnRH output is not fully understood, but it is generally accepted that the removal of an inhibitory effect of opioids allows an enhanced turnover of catecholamines (NA & A) which apparently influence the release of GnRH (Kalra, 1982; Kalra & Kalra, 1983). The mechanisms by which endogenous opioids exert their inhibitory effect on catecholamines is still unclear (Kalra, 1981). However, the effects at the opiates have been localized to the MPOA and the MBH, and may involve modulatory changes in catecholamine neurotransmission (Kalra, 1981).

c. The involvement of catecholamines during the neural critical period:

Several studies have demonstrated that an enhanced neurotransmission, in particular of catecholamines (DA, NA & A) which apparently occurs

during the neural 'critical period', participates in the output of GnRH and hence the LH surge and ovulation (Weiner & Ganong, 1978; Barraclough & Wise, 1982; Kalra & Kalra, 1983; Barraclough et al., 1984). However, the many neuropharmacological studies that have been attempted in order to determine a functional role for catecholamines in the regulation of LH release are contradictory and to date, a clear concept pertaining to the involvement of a particular catecholamine cannot be put forward.

#### Dopamine:

A large number of studies have strongly suggested that the tubero-infundibular dopamine system participates in the output of GnRH (Lichtensteiger, 1970; Hökfelt & Fuxe, 1972; Lichtensteiger & Keller, 1974). Cell bodies of the tubero-infundibular tract are mainly located in the arcuate nucleus and the axon terminals project to the ME (Ajika & Hökfelt, 1973 & 1975). Evidence that dopamine in the ME regulates GnRH output by its inhibitory role, came from a study in which the rate of decline in the catecholamine concentrations was measured after blockade of tyrosine hydroxylase (the rate-limiting enzymes for DA and NA) by  $\alpha$ -methyl-p-tyrosine ( $\alpha$ MPT) (Löfström, 1977). It was found that turnover of dopamine was low at proestrus when compared with the other days of the cycle and Löfström implied that disinhibition of DA on the GnRH nerve terminals induced the preoestrous output of GnRH. In confirmation of this study, the infusion of dopamine into the IIIrd ventricle (to avoid the blood brain barrier) of proestrous rats prior to the 'critical period' blocked ovulation. This inhibitory role of dopamine is not generally accepted since in contradiction with the above study, the intraventricular infusion of dopamine into ovariectomized oestrogen-primed rats showed a stimulatory effect on LH release and it was proposed that dopamine at the ME stimulates GnRH output

(Vijayan & McCann, 1978). More recently, it has been shown that the dopamine incerto-hypothalamic tract, the cell bodies of which originate in the All group of the thalamus and project their axon terminals to the zona incerta (ZI) of the hypothalamus, appears to stimulate the output of the LH surge and consequently ovulation (Mackenzie et al., 1984). In this study blockade of ovulation by pentobarbitone treatment was reinstated if DA was stereotaxically infused into the IIIrd ventricle before the 'critical period'. Similarly, a dopamine antagonist, haloperidol, infused into the zona incerta of proestrous rats prevented ovulation. Furthermore, in this study, using an ovariectomized and oestrogen-primed rat model, LH release was stimulated when dopamine was placed stereotaxically into the zona incerta.

#### Noradrenaline and adrenaline:

The first evidence that catecholamines (NA & A) might be involved in the regulation of the preovulatory surge of LH came from a study in which it was demonstrated that dibenamine (i.v.) and SKF501 (i.p.) (both  $\alpha$ -adrenergic receptor antagonists) blocked ovulation when administered to cyclic rats (Everett et al., 1949; Sawyer et al., 1950). In confirmation of this study, intraventricular infusion of NA and A prior to the 'critical period' induced ovulation in pentobarbital blocked rats (Rubinstein & Sawyer, 1970). Several reports have appeared to demonstrate that central catecholamines are involved in LH release since the first evidence of  $\alpha$ -receptor involvement in the output of gonadotrophins was reported by Everett & Co-workers (1949). An autoradiographic study showed that tritiated oestradiol was concentrated in the nuclei of the cell bodies of catecholamines neurones at the brain stem (Sar & Stumpf, 1981). This implies that genomic activation induced by oestrogen might be operative at the catecholamine cell body level. Subsequently, the

idea was accepted that catecholamines have a mediatory role for oestrogen in eliciting the positive feedback response of LH (reviewed by Kalra & Kalra, 1983; and Barraclough et al., 1984). During the critical period catecholamines, mediating the positive feedback response to oestrogen, may provide a stimulatory effect on the preovulatory surge output of GnRH which, in turn regulates the LH output from the anterior pituitary. Neurotoxins such as 6-hydroxydopamine, when stereotaxically injected into the ventral noradrenergic bundle of the MPOA, disrupted the catecholamine system and this led to blockade of ovulation (Beneditti et al., 1976; Martinovic & McCann, 1977; Nicholson et al., 1978; Hanckle & Wuttke, 1979). With the advent of microdissection techniques and more sensitive catecholamine assays, it has recently been possible to measure catecholamines in discrete areas of the brain. A considerable increase in NA content of the rat suprachiasmatic nucleus was found during the afternoon of proestrus as compared to the morning. Furthermore, in this study the median eminence showed an increase in NA content from dioestrous to proestrous stage while dopamine remained at basal level during the oestrous cycle (Selmanoff et al., 1976). Since the measure of turnover of a neurotransmitter seems to be a more sensitive index of neuronal activity (Costa & Neff, 1977; Weiner, 1974), in another study using  $\alpha$ MPT, changes in catecholamine turnover were measured in discrete areas of the rat brain on dioestrus and proestrus (Rance et al., 1981). It was observed that a significantly higher NA turnover in the medial preoptic area (MPOA) occurred during the proestrous afternoon and furthermore, a significant increase in NA turnover in the median eminence occurred at a later time concomitant with the LH surge. Rance et al suggested that increased NA turnover in the MPOA might be the neural mechanism underlying the neural 'critical period'. However, a further study concerning NA turnover in pentobarbital treated proestrous

rats did not support this suggestion since increased turnover of NA in the MPOA was found in pentobarbital blocked animals which was similar to that observed in control animals (Rance & Barraclough, 1981). This suggestion is apparently supported by the observation that neurotoxin - (6-hydroxydopamine) induced degeneration of NA axons or lesions of the ventral noradrenergic bundle lead to the loss of ovulation. However, this loss is only temporary since ovulation returns within three weeks (Clifton & Sawyer, 1979). An explanation which has been suggested is that NA axons regenerate after 2-3 weeks. It is equally possible that adrenaline axons were temporarily damaged and became operative at a later time, since the distribution of adrenaline axons is in parallel to that of noradrenaline axons (reviewed by Moore & Bloom, 1979). An overview of the anatomical studies indicates that the noradrenaline system in the rat brain is composed of two cell groups located at two sites in the brain stem (a) locus coeruleus (b) lateral tegmental. Both of these sites contain NA cell bodies that project axons via the ventral noradrenergic bundle to nearly all discrete areas of the hypothalamus. However, noradrenergic input to the hypothalamus comes largely from lateral tegmental brain stem neurones (reviewed by Moore & Bloom, 1979). Although, in the past, there has been disagreement over the existence of central adrenaline neurone systems due to the lack of techniques which could distinguish adrenaline from other catecholamines, recent enzymatic and immunohistochemical studies by Saavedra et al (1974b) and Hökfelt et al (1974) respectively, have revealed their existence. The enzymatic study measured the activity of phenylethanolamine-N-methyltransferase (PNMT) (the enzyme which converts NA to A, EC 2.1.1.28) in the rat brain nuclei. It was found that the PNMT activity was unevenly distributed both within the brain stem nuclei and the hypothalamic nuclei. The greatest PNMT activity

was measured in two areas of the brain stem, A1 and A2 which largely contain the catecholamine nerve cell bodies (Dahlström & Fuxe, 1964). Moreover, high PNMT activity was present in the mediobasal hypothalamus. With the immunohistochemical technique for PNMT which agrees well with the enzymatic technique it has been found that the enzyme is distributed roughly in parallel with what has been earlier considered to be a noradrenergic pathway (Swanson & Hartman, 1975). This raises the possibility that mechanisms attributed to NA in the brain may be exerted in part by adrenaline. Based on the enzymatic and immunohistochemical findings it is well accepted that the perikarya of adrenergic neurones are situated in the brain stem, in particular the lateral reticular formation from which axons project to the medial preoptic area (MPOA), perifornical area (PFA) and the arcuate-median eminence area (MBH).

Evidence that adrenaline may be a more potent catecholamine involved with the LH surge came from a series of studies. Thus an intraventricular infusion of adrenaline into reserpine or pentobarbital-blocked rats was more successful than dopamine and noradrenaline in reinstating the preovulatory LH surge (Rubinstein & Sawyer, 1970) (reserpine, a depleter of catecholamine (CA) from the storage granules by inducing a slow release of CA from the granules by inhibiting  $Mg^{2+}$ -ATP dependent granular uptake of CA]. In support of this study, it has been demonstrated that the stereotaxic infusion of adrenaline into the 3rd ventricle of ovariectomized oestrogen-progesterone primed rats in stimulating the LH output was more potent than DA & NA (Vijayan & McCann, 1978). Recently, several reports have appeared which implicated a critical role of adrenaline in the control of the LH surge. Among these, a study involving a selective inhibitor of adrenaline biosynthesis, SKF64139

(dichloro-tetrahydroisoquinoline) when administered (i.p., 50mg/kg) to long-term ovariectomized oestrogen-progesterone treated rats, 5-6h before the expected LH surge, completely blocked the output of the LH surge (Crowley & Cass-Terry, 1981). Furthermore, in another study to characterize further the role of central adrenaline in the neural regulation of the surge output of gonadotrophins (LH and prolactin) it was indicated that central adrenaline mediated the positive feedback response of ovarian hormones on LH secretion but not the oestrogen-induced increase in prolactin (Crowley et al., 1982). In parallel to the above studies, an investigation pertaining to indoleamines showed that p-chlorophenylalanine (PCPA), a potent inhibitor of tryptophan hydroxylase, blocked the LH surge induced by oestrogen to ovariectomized rats which was reinstated by the injection (i.p.) of 5-hydroxytryptophan (5-HTP) at 100h of the expected day of the LH surge (Coen & Mackinnon, 1979). However, neurotoxin induced lesion of the 5-hydroxytryptamine (5HT) projections from the raphe nuclei were consistent with a normal occurrence of the LH surge, which contradicted the earlier suggestions that 5HT plays an essential role in the output of the preovulatory LH surge (Coen et al., 1983). This paradox was resolved from the observations by Coen et al (1983) that PCPA inhibited the activity of PNMT and hence the adrenaline concentration was affected. Therefore, the possibility arose that PCPA might be blocking the LH surge through its influence on the adrenaline system. In confirmation of this study, the measurement of adrenergic activity based on the rate of decline of adrenaline 2h after injection (i.p., 50mg/kg) of SKF 64139 suggested that adrenergic activity increased in the MBH of adult cyclic rats during the neural critical period and furthermore, it was inhibited by pentobarbital as the preovulatory surge concentration of LH and ovulation (Mackinnon et al., 1983). Another investigation using acutely

ovariectomized oestrogen-primed rats showed that the increased adrenergic activity at the MBH was dependent on oestrogen, such an effect was not seen in the MPOA (Mackinnon et al., 1985).

In a previous study of ontology of the oestradiol benzoate (OE<sub>2</sub>) stimulated gonadotrophin (LH) and prolactin surge, it was found that this neuro-humoral event could be elicited in 21-day-old female rats but not in 16-day-old females or males at any age (Puig-Duran & Mackinnon, 1978a,b). These ontological studies are supported by others which indicated that in female rats the different nervous structures located in the anterior hypothalamic area involved in the positive feedback effect of ovarian steroid mature at about 22-days of age (Ojeda et al., 1971; Caligaris et al., 1978, Moguilevsky et al., 1981; Moguilevsky et al., 1982).

#### Aims:

In the present work I have used the steroid-stimulated infant rat as an experimental model (Puig-Duran & Mackinnon, 1978a) to test whether or not the increase of adrenaline concentration in the MBH, which is temporarily related to the output of gonadotrophin in adult female rats (Sheaves et al., 1985a) also occurs in OE<sub>2</sub>-stimulated infant females and furthermore, to characterize its possible involvement in the sexually differentiated positive feedback response to oestrogen. In addition, since adrenaline is synthesized from noradrenaline in various brain areas (Ciaranello et al., 1969) by the specific enzyme, phenylethanolamine-N-methyltransferase (Pohorecky et al., 1969), the activity of this enzyme in the MBH and MPOA at pertinent times after the initial injection of oestradiol benzoate was also investigated.



## Chapter Two

### GENERAL MATERIALS AND METHODS

The general materials and methods which have been used throughout this work are described in this chapter.

#### Animals

Male and female Albino Wistar rats were purchased as adults from Charles River, Kent, and when necessary mated in our animal house. The pregnant mothers were identified by palpation and caged individually until delivery. In our colony, parturition usually occurred on either day 21 or 22 of gestation. The day of birth with respect to our litters was designated day 0 and pups were culled within 24 hours so as to restrict the litters to a maximum of eight. Since previous observations had shown that weaning does not affect the gonadotrophin response to an oestrogen stimulus, weaning took place at 21 days of age. All rats were maintained under conditions of controlled lighting (lights on: 0600-2000h) and temperature ( $22\pm 2^{\circ}\text{C}$ ) and had free access to diet FFG [Dixon & Sons Ltd., Ware] and tap water. Immature rats of 16-25 day-old were used throughout this investigation. This chapter has been divided into four sections namely section 'A', 'B', 'C' and 'D'. Methods of measuring protein are described in the appendix.

#### SECTION 'A'

#### RADIOIMMUNOASSAY OF LUTEINIZING HORMONE

##### 1. Introduction

In a radioimmunoassay (RIA) minute quantities of a protein hormone are measured. This technique exploits the immunological properties

of the hormone to be measured and its competition with labelled hormone for a fixed number of binding sites on the antibody. This was introduced by Berson et al., (1956) when they observed that plasma from patients receiving insulin could bind insulin labelled with  $^{131}\text{I}$ . They found that such a reaction was of an antigen-antibody nature and the component with antibody properties was identified as gamma-globulin. In vitro studies demonstrated that if unlabelled insulin was added to labelled insulin, competition between the two occurred for binding sites on the antibody, furthermore competition was found to be quantitative (Yalow & Berson, 1960). This reaction formed the basis of radioimmunoassay technique. Subsequently, a simple method of labelling tyrosine residues in a protein with  $^{125}\text{I}$  or  $^{131}\text{I}$  using chloramine-T as oxidant (Greenwood et al., 1963) enabled this technique to be routinely used for measuring hormones in biological fluids or tissues. The first RIA for rat luteinizing hormone (LH) was reported by Monroe et al. in 1968. Since this technique utilized highly purified rat LH for iodination and purified rat LH for immunization, this assay is known as rat-rat LH radioimmunoassay (RR rat LH RIA). This assay was much more sensitive than previously existing biological assay for quantifying LH levels. Although the first radioimmunoassay was capable of measuring LH levels in both pituitary extracts and samples of serum, it was still not possible to measure sequential samples of sera from the same rat since the volumes required were too great ( $500\mu\text{l}$ ). A highly sensitive assay was subsequently developed by Niswender et al. in 1968 known as ovine ovine rat radioimmunoassay (OO rat RIA) which ensured that smaller volumes of sera could be measured. However, this microassay has certain limitations, at least  $200\mu\text{l}$  of serum is the absolute requirement of this assay. In certain instances, the volume of fluid available may be severely limited because of: (a) the need for specimens to be assayed in duplicate;

(b) the physiological disruption attendant upon the collection of relatively large amounts of body fluids and, (c) lack of sufficient volume in collections taken from very small animals. These difficulties were solved when Naftolin and Corker (1971) scaled down the original RIA by Niswender et al. (1968) and made it possible to measure LH using samples of as little as 10 $\mu$ l. This assay is known as the ultramicromethod for the measurement of LH by RIA and this has been used in the present laboratory since it was set up in 1970.

## 2. Reagents

### 2.1 Buffers

(a) Phosphate Buffered Saline (PBS) was made from 10 litres of 0.14M NaCl containing 68.6ml 0.5M NaH<sub>2</sub>PO<sub>4</sub>, 137ml 0.5M Na<sub>2</sub>HPO<sub>4</sub>, 1g thimersol, an antibacterial agent [Sigma Chemical Co. Ltd., U.K.] and the pH adjusted to 7.0 if necessary.

(b) 0.5M Phosphate Buffer for protein iodination 0.5M NaH<sub>2</sub>PO<sub>4</sub> and 0.5M Na<sub>2</sub>HPO<sub>4</sub> were mixed in an approximate ratio of 1:8 to give a final pH of 7.5.

(c) 0.05M Phosphate Buffer 0.05M Na<sub>2</sub>HPO<sub>4</sub> and 0.05M NaH<sub>2</sub>PO<sub>4</sub> were mixed in an approximate ratio of 1:8 to give a final pH of 7.5.

(d) Special Buffer was made from ethylenediamine tetra-acetic acid [EDTA, a chelating agent; BDH, Dagenham], 0.05M in PBS, to which was added normal rabbit serum (NRS) to give a final dilution of 1:300 of the NRS.

(e) Egg White Solution (EW) 1% (W/V) EW was made from 990ml PBS, 10ml 1:100 thimersol and 10g egg white. 5% (W/V) EW was made from 99ml PBS, 1ml 1:100 thimersol and 5g egg white.

2.2 Protein for iodination purified ovine LH (OLH, LER 1056-C2) was kindly donated by Professor L. E. Reichert Jr. It had a biological

potency of 1.71xNIH-LH-S1 Units/mg by ovarian ascorbic acid depletion (OAAD) test and negligible, if any, TSH or FSH contamination. This material was dissolved in distilled water to give a concentration of  $1 \mu\text{g}/\mu\text{l}$  and  $5 \mu\text{l}$  aliquots were stored in autoanalyzer vials and frozen at  $-20^{\circ}\text{C}$  until ready to use.

### 2.3 Antibodies

(a) Antiserum to ovine LH (AOLH). This was raised in rabbits and kindly donated by Professor G. D. Niswender. The lyophilised material (AOLH, GDN-15) was dissolved in distilled water to give a 1:400 dilution and stored in 1.0ml aliquots at  $-20^{\circ}\text{C}$ . When needed, the antiserum of the 1:400 dilution was further diluted to 1:32,000 with special buffer.

(b) Anti-Rabbit Gamma Globulin (ARGG). This was raised in donkeys and obtained from Burroughs Wellcome [Beckenham, Kent]. It was used as the precipitating antibody at a dilution of 1:16 in PBS.

### 2.4 Standards

A standard ovine LH preparation, prepared from pituitary (NIH-LH-S13) was obtained from the National Institute of Arthritis, Diabetes, Digestive Diseases & Kidney (NIADDK). The biological potency was estimated as  $0.95 \times \text{NIH-LH-S1 units per mg}$  by OAAD test and negligible if any, TSH or FSH contamination. The lyophilized standard was dissolved in 1% EW to concentrations of 0.25, 1.0, 2.0, 4.0, 8.0, 16.0 and  $32.0 \mu\text{g}/\text{l}$ .

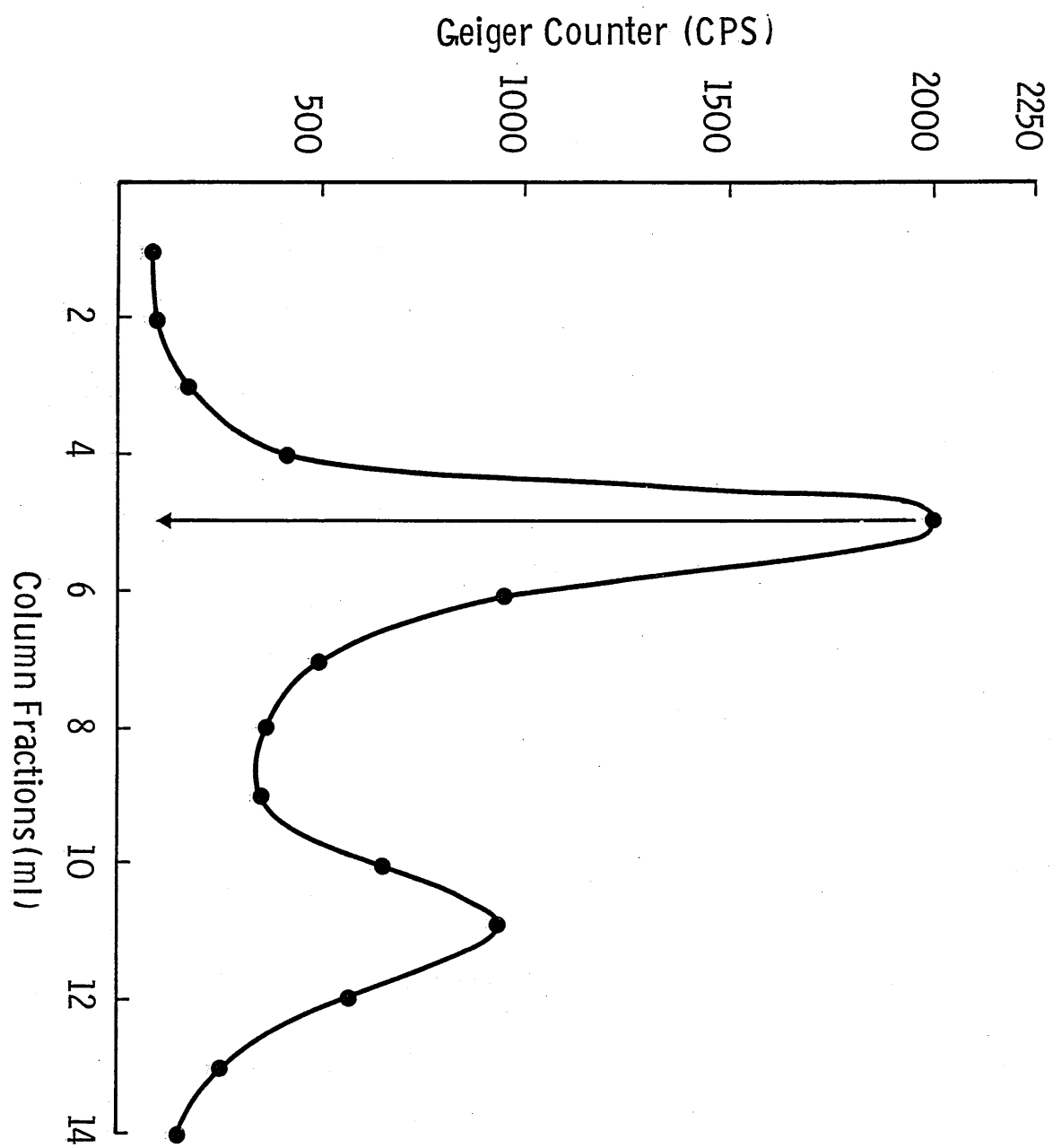
Aliquots of  $50 \mu\text{l}$  for each dilution were stored in colour-coded LP2 tubes (Luckhams Ltd., Sussex) and frozen at  $-20^{\circ}\text{C}$  until use.

2.5 Radio-iodination of LH protein. Radioiodination of ovine LH protein was performed by the method of Greenwood et al. (1963). All iodination procedure was carried out inside an enclosed hood (which was accessible through armlets and which had an extractor fan), housed in a special 'iodination room'. The reaction vessel was an autoanalyzer vial, into which all reagents were injected.

A 5  $\mu$ l aliquot of OLH was buffered with 50  $\mu$ l 0.5M phosphate buffer, followed by 1mCi of sodium- $^{125}$ I [specific activity: 16.8 mCi/ $\mu$ g. Amersham International, U.K.]. 20  $\mu$ l of 0.0036M chloramine-T [BDH, Dagenham] in 0.05M phosphate buffer was added in order to oxidize the iodide and enable the  $^{125}$ I to attach the tyrosine residues of the protein. The contents of the vessel were mixed for 30 sec. and the reaction terminated by the addition of 100  $\mu$ l of 0.0126M sodium metabisulphite [BDH, Dagenham] diluted in 0.05M phosphate buffer. The iodination mixture was loaded on to a column (1cm x 12cm) of Sephadex-G75 [Pharmacia Uppsala, Sweden] using a pasteur pipette which had been pre-rinsed in 5% EW so as to minimize the absorption of protein on the glass. The sephadex had been pre-soaked in 0.05M phosphate buffer, 1ml of 5% EW passed through the column in order to minimize protein loss from the iodination mixture by adsorption. The protein was eluted with 0.05M phosphate buffer and 1ml fractions were collected in glass tubes [PTF104, Gallenkamp], each containing 1ml of 5% EW. The radioactivity in the fractions was measured with an X-ray Mini Monitor [Mini-Instruments, London] and revealed 2 peaks of radioactivity, an early peak of protein and a later peak of free iodine (Fig.2). The fraction of the initial peak with the most radioactivity was selected since it was consistently identifiable. This fraction was usable for at least 4-5 weeks if it was kept frozen at -20°C. For subsequent use in the assay, the labelled OLH was further diluted in 1% EW to give 5,000-7,000 CPM/50  $\mu$ l in a Berthold Auto-Gamma Counter [LB MAG310, Germany].

**2.6 Assay Method.** All manipulations were carried out at 4°C on ice. Each component of the assay was added in a 50  $\mu$ l volume. Serum samples were dispensed into disposable plastic LP2 tubes using a Finnpiptette [Labsystems, Finland] while other reagents were dispensed using repettes [Jencone Scientific Ltd., Hemel Hempstead].

Fig. 2. Separation of  $^{125}\text{I}$ -labelled ovine LH from  $^{125}\text{I}$ -iodide and other low molecular weight reactants, on a sephadex G-75 column. The arrow indicates the column fraction containing labelled LH which was collected for subsequent radioimmunoassays (see text). The second peak indicates the column fraction containing  $^{125}\text{I}$ -iodide and other low molecular weight reactants.



Each assay included:

- (i) Three 'total count tubes', which contained 50 $\mu$ l of labelled OLH only, to record the number of counts added to every tube in the assay;
- (ii) Reference tubes without antibody (Nil AOLH) run in triplicate, to assess the amount of labelled OLH precipitated in the absence of the antibody;
- (iii) Reference tubes without unlabelled hormone (Nil OLH), run in triplicate, to assess the maximum amount of labelled OLH that bound to AOLH in the absence of competing unlabelled LH;
- (iv) A standard curve, run in triplicate;
- (v) Aliquots of pooled rat serum samples, run in triplicate, to assess the inter-assay variation;
- (vi) Unknown samples run in duplicate.

The following timetable was followed:-

Day 1 - Unknown serum samples (50 $\mu$ l) were dispensed into LP2 tubes and 1% EW and AOLH were added to these tubes and to the standards and serum pools; the Nil OLH tubes received an aliquot of 1% EW instead of unlabelled hormone, and the Nil AOLH tube received special buffer in place of AOLH. All tubes were then whirlmixed briefly and left at 4°C for 24h.

Day 2 - 50 $\mu$ l of labelled OLH containing approximately 5,000-7,000 C.P.M. was added to each tube; the tubes were whirlmixed briefly and left at 4°C for 48h.

Day 3 - No manipulations were performed.

Day 4 - 50 $\mu$ l of ARGG was added to all tubes except the 'total counts tubes'; the tubes were whirlmixed briefly and left at 4°C for 24h.

Day 5 - All tubes, except the 'total count tubes' were spun at 15,00g in a refrigerated centrifuge at 4°C for 45 min. The supernatant containing the unbound fraction, was aspirated and discarded, and the



precipitate left in the tube was counted in a Berthold Auto-Gamma counter for 2 minutes.

2.7 Calculations. The counts in the Nil AOLH tube were subtracted from the counts in all other tubes and their individual counts were expressed as a percentage of the Nil OLH tube (maximum bound). The standard values were then plotted on a semi-logarithmic paper to give a standard curve from which the value of unknowns and pools were read off this curve (Fig.3). Duplicates of unknown samples in the same assay were with 10% of each other otherwise they were discarded and the assay repeated.

## 2.8 Reliability Criteria

Precision was estimated from the coefficient of variation which is calculated by expressing the standard deviation of a group of assay results of the same sample as a percentage of the mean value. There are two kinds of coefficients of variation,

(a) Inter-assay coefficient of variation. This was obtained by estimating the same sample, at least, in 9 separate assays.

(b) Intra-assay coefficient of variation. This was obtained by measuring, at least, 8 aliquots of the same sample in the same assay.

Inter- and Intra-assay coefficients of variation are as follows :-

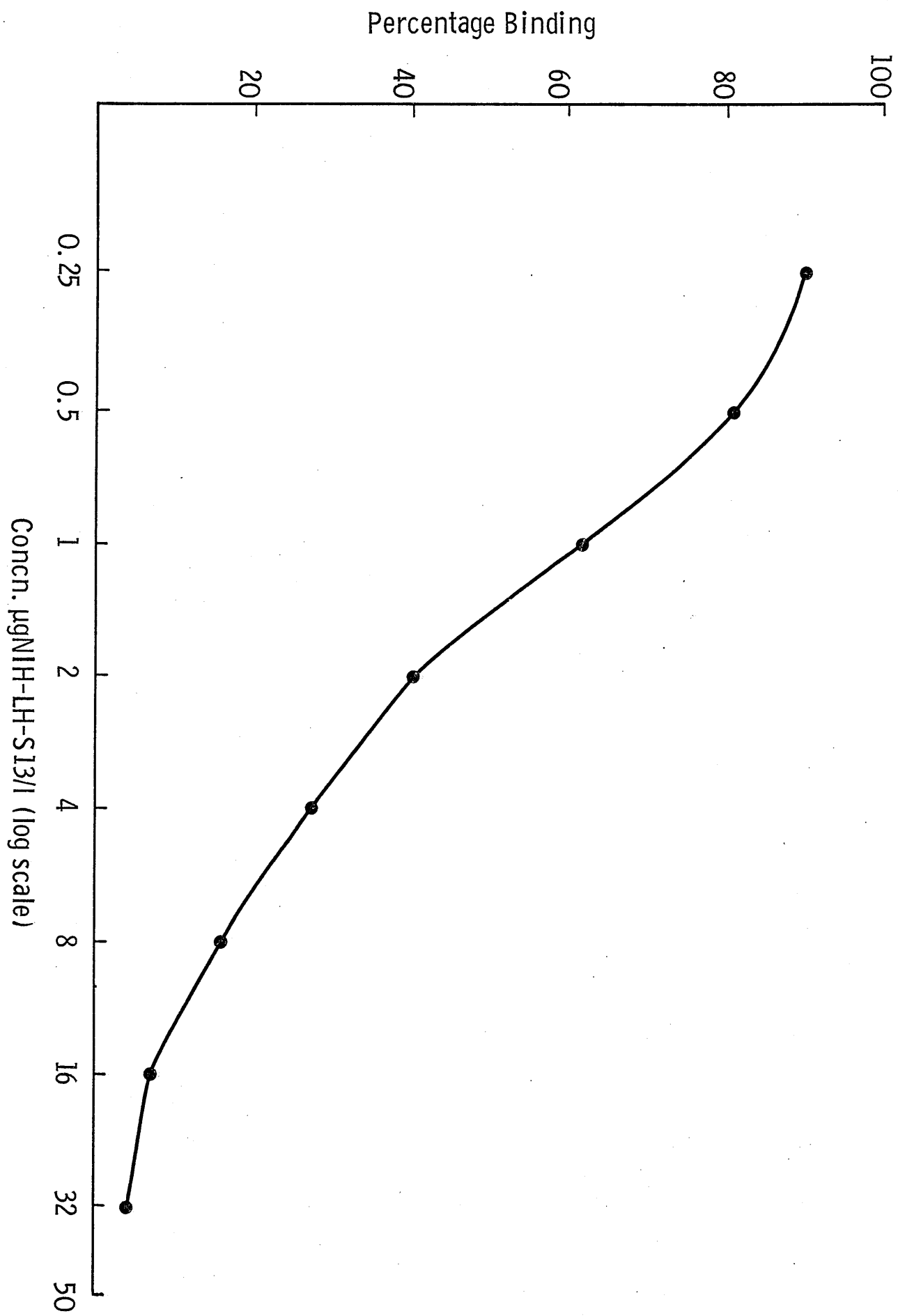
Inter-assay coefficient of variation:

	No.	Mean ( $\mu\text{g}/\text{l}$ )	Standard deviation	Coefficient of variation
Pooled Serum	1	0.60	0.050(n=10)	8.25%
" "	2	2.93	0.377(n=10)	12.79%
" "	3	18.20	1.653(n=9)	9.08%

Mean of coefficients of variation = 10.04%

Fig.3. A typical standard curve obtained by using NIH-LH-S13 standards (0.25 - 32  $\mu\text{g}/1$ ).

Each point is a mean of 3 determinations.



Intra-assay coefficient of variation:

	No.	Mean ( $\mu\text{g}/\text{l}$ )	Standard deviation	Coefficient of variation
Pooled serum	1	0.57	0.10(n=10)	7.53%
" "	2	3.08	0.31(n=8)	10.06%
" "	3	19.52	0.83(n=10)	4.25%

Mean of coefficients of variation = 7.28%

2.9 Sensitivity and Specificity. According to the 'Approved Recommendation on Quality Control in Clinical Chemistry' (Büttner et al., 1980), sensitivity is defined as twice the standard deviation ( $2 \times \text{S.D.}$ ) of the blank values. On the basis of this criterion and considering the reference tubes with Nil AOLH the sensitivity resulted in approximately  $0.25 \mu\text{g}$  NIH-LH-S13/1 which gave a displacement of bound counts of about 10% (Fig. 3). This was considered to be the smallest amount of hormone which could be reliably measured. Values lower than this were considered to be non-detectable (ND). Serum samples in which the LH concentration was found to be too high to be read off the standard curve were considered to be greater than  $32 \mu\text{g}/\text{l}$  [ $>32$ ]. The assay is considered to be highly specific for LH since the antibody does not cross react with either TSH or FSH (Niswender et al., 1968).

## SECTION 'B'

### MICRODISSECTION OF DISCRETE HYPOTHALAMIC AREAS OF THE BRAIN

#### 1. Introduction

When neurochemical studies are performed on brain samples from small animals, it is difficult to excise well defined areas with good reproducibility by visual free-hand dissection. However, a micropunch technique developed by Palkovits (1973) has solved this difficulty and enables the removal of specific brain areas, nuclei, tracts and other sub-divisions of nuclei. This technique has been successfully applied in the determination of brain amines; serotonin (Saavedra et al., 1974a), noradrenaline and dopamine (Palkovits et al., 1974) and enzymes, phenylethanolamine-N-methyltransferase (PNMT) (Saavedra et al., 1974b) and serotonin synthesizing enzymes (Saavedra, 1977). Since the accurate measurements of catecholamines and PNMT activity present in discrete hypothalamic areas were the objectives of this study, the micropunch technique was used throughout. If post-mortem changes in brain amines and the loss of PNMT activity are to be avoided, the tissue must be frozen as soon as possible after death. In the micropunch method, this is fulfilled because freezing of the brain is an essential requirement.

#### 2. The Micropunch Technique

This technique requires:

(a) Removal of the brain. Decapitation is the most practical method of killing (Palkovits & Brownstein, 1983) and this was adopted throughout the present work. Only four immature rats of the same sex were kept per cage [Dimensions: 15" x 10" x 7" (length x width x height)] and of these a maximum of two were used per decapitation session to

minimize stress effects. Rats were decapitated with large scissors and occipital muscles from the occipital bone were cut off to expose the foramen magnum. With bone nibblers introduced into the foramen magnum, two parallel cuts were made through the parietal bone sutures extending rostrally as far as bregma. The bone was then lifted forward and cut off at the point of attachment. It was important to keep the blade parallel to the skull in order to avoid any damage to brain tissue. The brain was then elevated at the rostral end and the optic nerves severed with small scissors. The brain was removed to a cold plate resting on ice, with ventral side of the brain lying uppermost. A cut was then made in the coronal plane at the rostral end of the optic chiasma, leaving the optic nerves intact. A second cut was made near the caudal end of the mammillary bodies. This portion of the brain which includes the whole hypothalamus was mounted on its caudal end on a microtome chuck onto which 200 $\mu$ l water had been pipetted. The chuck was immediately placed in solid CO<sub>2</sub> (dry ice). The whole procedure of decapitation, removal of the brain and placing on solid CO<sub>2</sub> was completed within 2 minutes. If the brain was not immediately required for sectioning, it was wrapped in cling foil and stored at -70°C.

(b) Sectioning frozen brains. The frozen brains mounted on their chucks were placed initially in a cryostat [Slee, London] at -12°C to equilibrate for at least 30 minutes, before sectioning. Individual chucks were then attached to the microtome and serial coronal sections (250 $\mu$ m) were cut. An anti-roll device consisting of a plastic plate attached to the microtome was used to flatten out the sections. The frozen sections were mounted on glass slides by secured partial thawing within the cryostat (by rubbing the under surface of the slide with a finger) and they were then rapidly refrozen. Two serial sections

rostral to the caudal end of the anterior commissure which approximately correspond to 7020-6570 $\mu$ m in the rat brain Atlas (König & Klippel, 1963) were collected for punching out MPOA. Further serial sections were taken caudal to the optic chiasma and until the pituitary stalk was seen. These were used for punching out the MBH. These sections correspond to approximately 4890-4230 $\mu$ m (König & Klippel, 1963).

(c) Removal of brain samples. With the use of a dissecting microscope [Carl Zeiss, Germany] a hollow needle punch with an internal diameter 0.85mm, a small metal spring device attached to the stylet which ensured release of tissue samples (Fig.4) brain samples were removed. The samples were placed on a slide lying on a dark plastic container filled with solid CO<sub>2</sub> thus avoiding any likelihood of sections becoming thawed.

### 3. Punching out of medial preoptic area and mediobasal hypothalamus

The MPOA was punched out bilaterally from each of the two rostral sections at a point half-way up the 3rd ventricle and 1mm lateral to it (Fig.5a). The MBH was punched out from 3 or 4 serial sections after the lateral recessus of the 3rd ventricle appeared (Fig.5b).

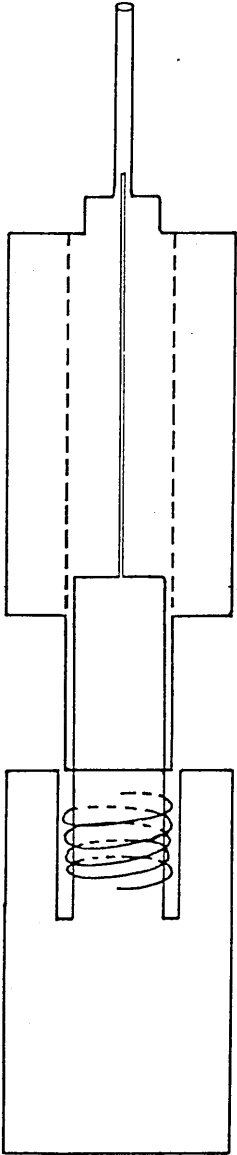
Fig.4. Diagrammatic representation of the:

- (a) stainless-steel punch equipped with a stylet (internal diameter, 0.85mm).
- (b) stainless-steel stylet equipped with a small spring.



a.

0.85mm



b.

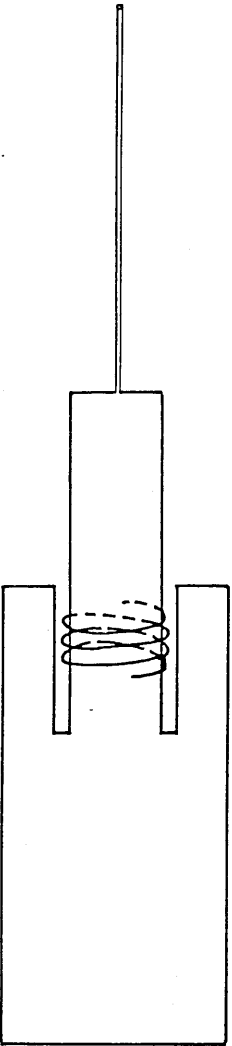


Fig.5a. Simplified diagram of a coronal section (containing anterior commissure) of the rat brain showing the location of the medial preoptic area (MPOA). The circle in the diagram represents the inner diameter of the dissecting needle (0.85mm).

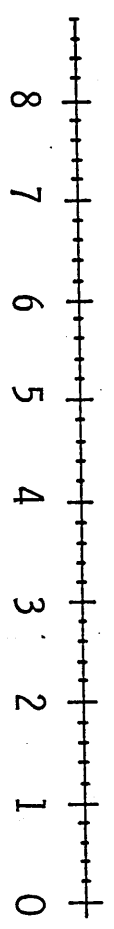
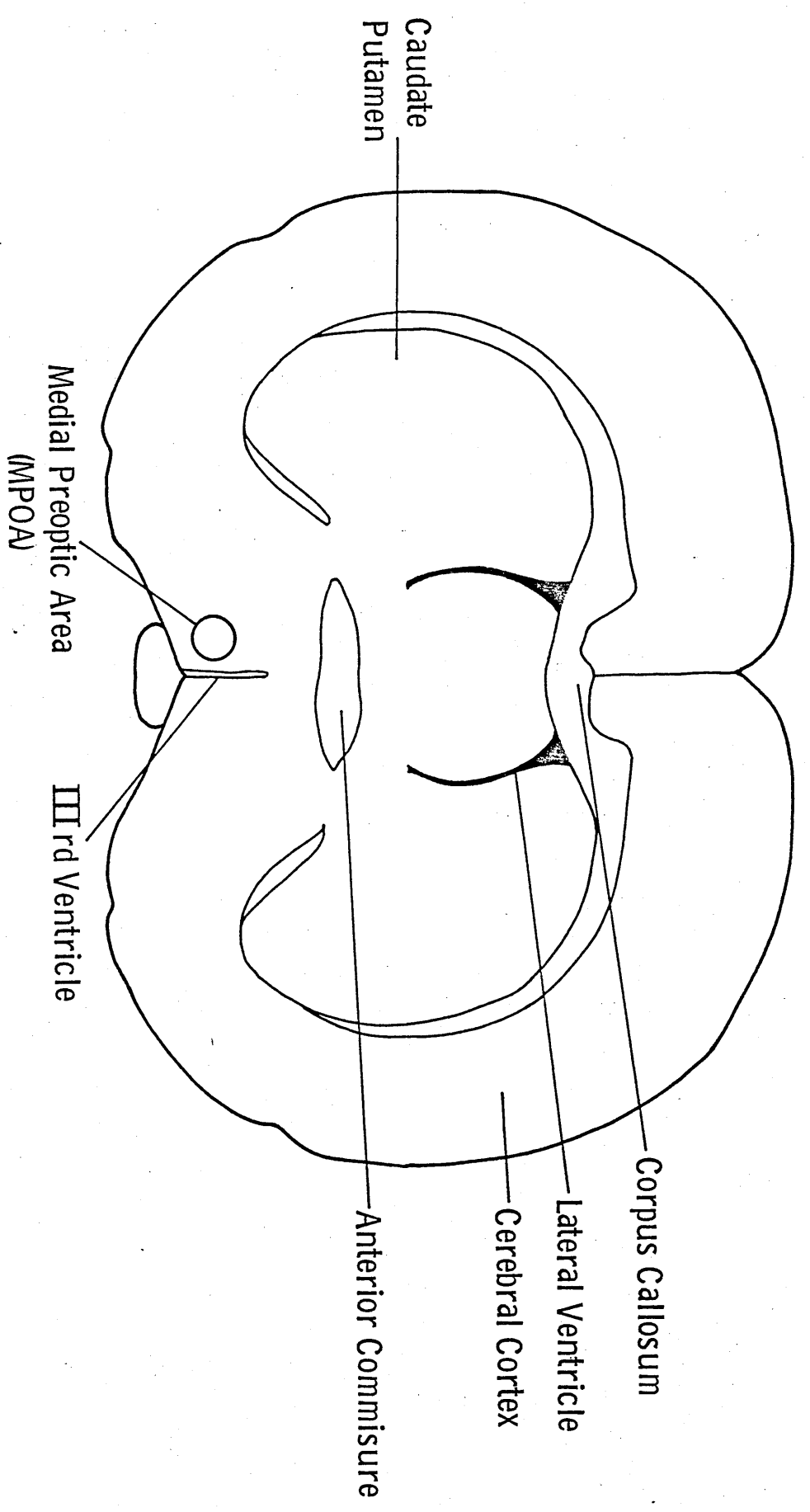
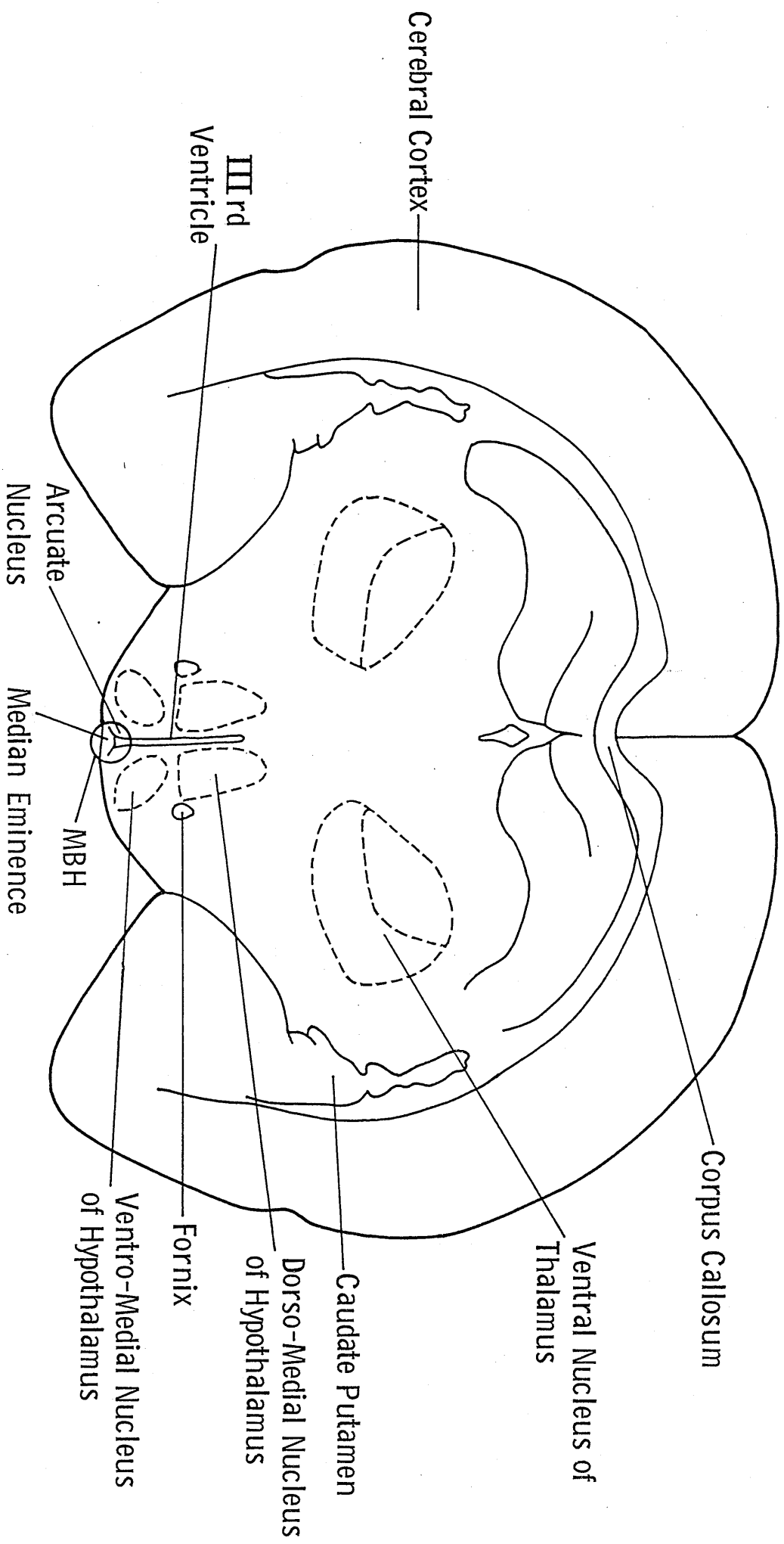


Fig.5b. Simplified diagram of a coronal section (containing lateral recessus of the IIIrd ventricle) of the rat brain showing the location of the medio-basal hypothalamus (MBH). The solid circle in the diagram containing arcuate nucleus-median eminence (MBH) represents the inner diameter of the dissecting needle (0.85mm).



## SECTION 'C'

### RADIOENZYMATIC ASSAY OF CATECHOLAMINES

#### 1. Introduction

A number of sensitive and specific radioenzymatic methods have been developed for the determinations of small amounts of catecholamines and other compounds carrying a catechol moiety. These methods are based on the enzymatic methylation of the catechols using catechol-o-methyl transferase (COMT) and s-adenosyl-methionine (SAM) as the methyl donor.

The original assay reported by Engelman et al. (1968) for measuring total  $\beta$ -hydroxylated catecholamines in urine and plasma stimulated much interest because of the 10-fold increase of sensitivity over conventional spectrofluorometric methods. Thus Nikodijevic et al. (1969); Engelman and Portnoy (1970); Passon and Peuler (1973), improved the original method by introducing a thin layer chromatography (TLC) step for the separation of noradrenaline (NA) from adrenaline (A); however, it could only be applied to tissues containing  $\beta$ -hydroxylated catecholamines. Since  $\beta$ -hydroxylated catecholamines (NA & A) and dopamine (DA) are equally good substrates for COMT, (Guldborg and Marsden, 1975), methylated products of both amines would be recovered. Thereafter, other workers made attempts to separate these amines. Thus Coyle and Henry (1973) reported the use of organic extraction together with a selective periodate oxidation of NA to separate it from DA. Similarly, Cuello et al., (1973) reported the use of COMT for the enzyme radiochemical assay of dopamine together with paper chromatography for the separation of dopamine from noradrenaline and

adrenaline. In subsequent years, various modifications were made to improve the method and to increase the sensitivity and specificity of the assay (Palkovits et al., 1974 ; Daprada and Zürcher, 1976; Gauchy et al., 1976; Weise and Kopin, 1976; Peuler and Johnson, 1977). Finally, Saller and Zigmond (1978), improved the method still further making it sufficiently sensitive to analyze small amounts of DA, NA, and A in either plasma or in tissue.

Currently, there are three main techniques available which are routinely used in various laboratories depending on the requirements to be met.

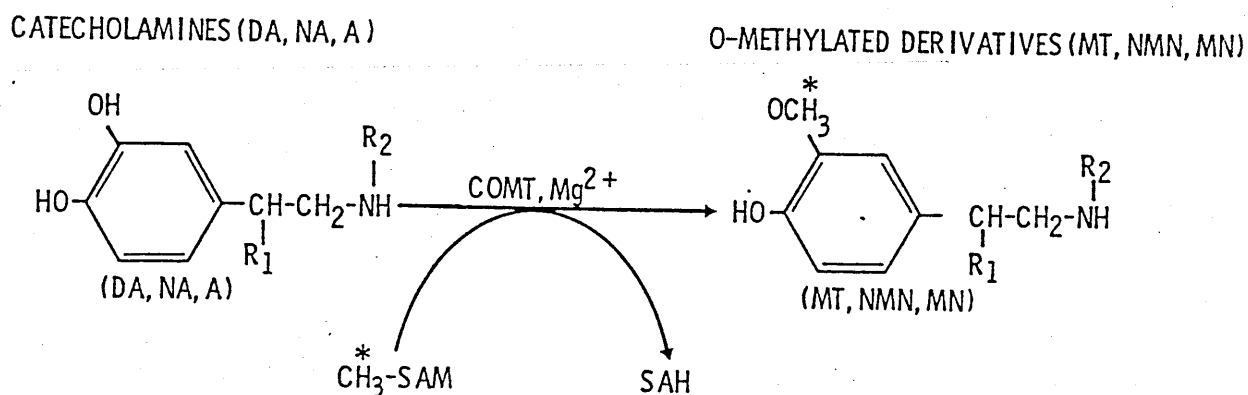
(a) High pressure liquid chromatographic method (HPLC). Several workers have used HPLC in order to separate the catecholamines and their respective derivatives (Refshauge et al., 1974; Mell and Gustafson, 1977; Kissinger et al., 1975; Erikson et al., 1977; Hansson et al., 1978; Imai and Tamura, 1978). With these methods measurement was achieved by ultra-violet or fluorometric or electrochemical detection. These HPLC methods, however, are of only limited sensitivity and cannot be used for samples containing relatively low amounts of catecholamines (e.g. certain brain areas and urine samples).

(b) Radioenzymatic assay with HPLC. This technique makes use of COMT and SAM as the methyl donor. The O-methylated derivatives of catecholamine (DA, NA & A) are separated by HPLC. Thus Appel et al. (1981) reported the determination of catecholamines with radioenzymatic assay together with HPLC.

(c) Radioenzymatic assay with TLC. This technique also makes use of COMT and SAM as the methyl donor, but the O-methylated derivatives are separated by TLC. Basically, (b) and (c) are of equal sensitivity, accuracy and specificity and therefore of equal choice. However, HPLC separation method is rapid and labour saving as compared to that of

TLC separation.

In the present study, the radioenzymatic assay of Saller and Zigmond (1978) was set up with certain modifications which will be mentioned whenever they are used in the assay. The assay is based on the enzymatic conversion of DA, NA and A to their derivatives in the presence of COMT using S-adenosyl-L-[methyl- $^3\text{H}$ ]methionine ( $^3\text{H}$ -SAM) as methyl donor. Solvent extraction of these derivatives and subsequent separation of methoxytyramine (MT), normetanephrine (NMN) and metanephrine (MN) was performed using TLC. The basic principle of this assay is diagrammatically shown below:



COMT, Catechol-O-methyl transferase

$^*\text{CH}_3\text{-SAM}$ , tritium labelled S-adenosyl methionine

SAH, S-adenosyl homocysteine

$\text{R}_1$  is -H for DA and MT, -OH for NA, NMN, A and MN

$\text{R}_2$  is -H for DA, MT, NA and NMN,  $-\text{CH}_3$  for A and MN

## 2. Materials and Methods

1000  $\mu\text{l}$  (1mCi)  $^3\text{H}$ -SAM (15 curie/mmol) supplied in dilute sulphuric acid/ethanol solution (9:1, v/v) was purchased from Amersham International, U.K. This solution was divided into 60 $\mu\text{l}$  aliquots in order to avoid multiple freezing and thawing. The aliquots were stored at  $-20^\circ\text{C}$  (decomposition was minimized when stored under these conditions;



Amersham International personal communication) until use. Under these conditions, SAM is stable for several months. Dimethyldichlorosilane, hydrochloric acid (HCl), magnesium chloride ( $MgCl_2$ ), perchloric acid ( $HClO_4$ ), ammonium sulphate  $[(NH_4)_2SO_4]$ , sodium metabisulphate ( $Na_2S_2O_5$ ), di-sodium hydrogen orthophosphate dihydrated ( $Na_2HPO_4 \cdot 2H_2O$ ), sodium di-hydrogen orthophosphate dihydrated ( $NaH_2PO_4 \cdot 2H_2O$ ), methanol, chloroform, toluene and acetic acid (1M), were all purchased from British Drug House (BDH), Dagenham, and were of Analar grade, dithiothreitol (DTT), boric acid, pargyline, potassium chloride (KCl), ethyleneglycol-bis-( $\beta$ -amino ethyl ether) N, N-tetra-acetic acid (EGTA), diethylene triamine penta acetic acid (DTPA), L-arterenol bitartrate (L-norepinephrine bitartrate), epinephrine bitartrate (adrenaline bitartrate), 3-hydroxytyramine hydrochloride (dopamine hydrochloride), normetanephrine, metanephrine, methoxytyramine, tris acetate, tris base (trizma), di-(2-ethyl hexyl) phosphoric acid (DEHP), albumin bovine fraction V (BSA) and 70% ethylamine were all obtained from Sigma Chemical Company, U.K. and were of highest obtainable grade commercially. 2,5-diphenyloxazole (PPO) and 1,4-bis(2-(5-phenyl oxazolyl)-benzene (POPOP) were obtained from Koch-light laboratories, U.K. All the solutions used in the present work were prepared with double-distilled water which was prepared by 2nd distillation of deionized distilled water (see Passon & Peuler, 1973). Silica gel plates (LK6DF, 20 x 20cm, 250 $\mu$ m thickness) were used for thin layer chromatography and were obtained from Uniscience Ltd., Cambridge. Catechol-o-methyl transferase (COMT) was prepared from rat liver, according to the following authors (Axelrod & Tomchick, 1958; Coyle & Henry, 1973; Cuello, 1979). Dialysis tubing (Visking 18/32) was obtained from medicell International Ltd., U.K. Glass tubes were purchased from Gallenkamp, U.K. Microlitre Hamilton syringes were supplied by Howe & Co. Ltd., U.K. Scintillation vial inserts

(MKII) and vials were obtained from Hughes & Hughes Ltd., Essex, U.K. Ultra-violet lamp, model UVSL-58 was supplied by Ultra Violet Products, U.S.A.

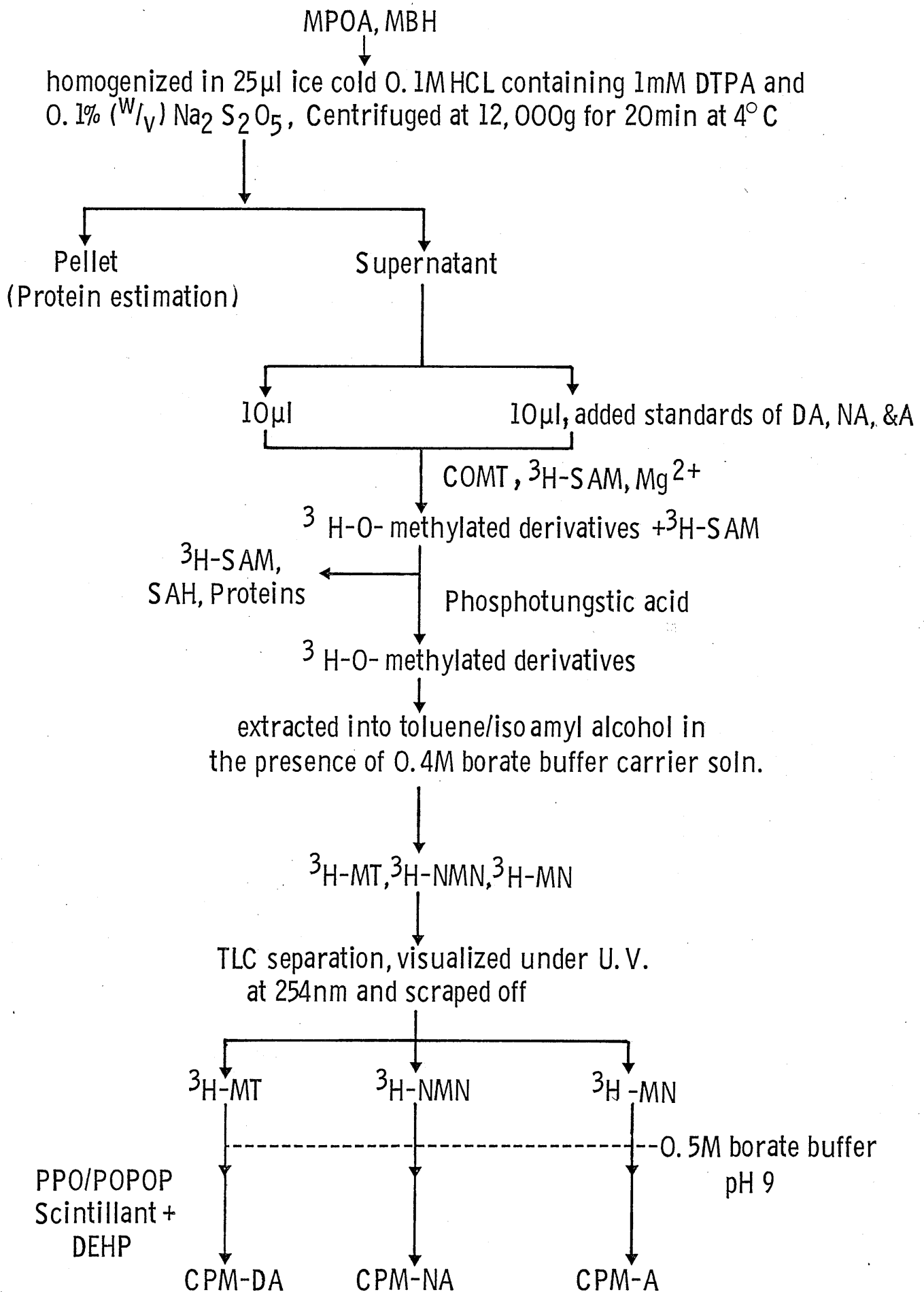
## 2.1 Preparation of Catechol-o-methyl transferase (COMT)

COMT was isolated by ammonium sulphate precipitation and the precipitate (30-45% fraction) collected as crude COMT preparation<sup>Coyle & Henry, 1973</sup>. Rat liver was homogenized in 4 volumes (4ml per 1g of liver) of isotonic KCl (1.19%, w/v) and filtered through glass wool in order to remove lipids. The filtrate was centrifuged at 78,000g for 1h at 4°C. The resultant pellet which contained broken cells and nuclear debris was discarded and the supernatant again filtered through glass wool to remove the residual lipids. 1M acetic acid was added to the supernatant dropwise to bring the pH down to 5.3. The preparation was left for 20 min. to equilibrate while being kept on ice. It was then spun at 16,000g for 20 min. at 4°C to separate the precipitate. The supernatant was removed and adjusted to pH 6.8 by adding a few drops of freshly prepared 0.5M sodium phosphate buffer (pH=7.0). Dry ammonium sulphate, 30% (w/v) saturation (16.4g/100ml of supernatant) was added to this supernatant, slowly, over a period of 10 min. Then left for 1h while being gently stirred on ice. The precipitated protein was removed after centrifugation (16,000g for 20 min) at 4°C and discarded. Dry ammonium sulphate (8.6g/100ml supernatant) was added to the supernatant to bring the saturation to 45% as described above. After centrifugation (16,000g for 20 min) at 4°C the supernatant was discarded and the resulting precipitate (30-45% fraction) dissolved in 0.1 volume (0.1ml per 1 gram of liver weight) of 0.1M sodium phosphate buffer (pH=7.0) containing 0.1mM DTT. In order to prevent the protein from frothing the precipitate was manually dissolved with the help of a sealed pasteur pipette. The preparation was then dialyzed overnight against three

changes of 500 volumes (v/v) of the redissolved 30-45% fraction of  $(\text{NH}_4)_2\text{SO}_4$  precipitate) of the same buffer. This step was taken in order to remove  $(\text{NH}_4)_2\text{SO}_4$  and other small molecules from COMT. The dialyzed product was centrifuged (16,000g for 20 min) at 4°C to remove precipitated proteins and the clear supernatant was obtained. Sufficient DTT was added to the supernatant to make the final concentration of 1mM and aliquoted into 100µl aliquots and stored at -20°C until use. Under these conditions, partially purified enzyme, COMT appears to be stable for several months. The specific activity of COMT could be measured according to McCaman (1965) by using 3,4 dihydroxy benzoic acid; in the present study, however, this was not performed, since it was more convenient to compare each enzyme preparation with the previous preparation in order to determine the optimum volume of the enzyme to be used. It was found that the activity of the enzyme was consistent from batch to batch provided that standard conditions are used as described above.

**2.2 Tissue extraction.** Extraction of catecholamines and the standard assay procedure are shown in a flow sheet (Fig.6). Brain samples taken from the MBH and the MPOA (as previously described) were homogenized in 25µl of 0.1M HCl containing 1mM DTPA and 0.1% (w/v)  $\text{Na}_2\text{S}_2\text{O}_5$ . Although Sallar and Zigmond (1978) used 0.2M  $\text{HClO}_4$  as an homogenizing medium for the extraction of brain tissues, 0.1M HCl was used in the present study. It has been shown by Gunne (1963) that the stability of biogenic amine, serotonin in perchloric acid extract is poor. Furthermore, serotonin was extracted from the brain samples in 0.1M HCl instead of 0.2M  $\text{HClO}_4$  (Saavedra, 1977). In the light of these observations, 0.1M HCl was used as a homogenizing medium, throughout 1.0mM DTPA was included in the homogenizing medium as a general chelating agent in order to prevent inference from heavy metal ions

Fig.6. Diagrammatic summary of the methods of tissue preparation,  
and estimation of catecholamines.



(e.g. those of Fe, Zn, Cu, Pb, Hg). It was thought that they may be introduced into the homogenate from glassware, traces from double-distilled water or as a contaminant in commercial reagents. Since sodium metabisulphite was found to be the most suitable antioxidant, this too was added to the homogenizing medium, in 0.1% (w/v) to prevent destruction of catecholamines during the homogenization and storage. 0.1M HCl containing 1mM DTPA was made as a stock solution and 0.1%  $\text{Na}_2\text{S}_2\text{O}_5$  was added just before extraction. The tissue was homogenized in 25 $\mu$ l ice-cold 0.1M HCl/DTPA/ $\text{Na}_2\text{S}_2\text{O}_5$  by means of a silanized glass micro-homogenizer with a right fitted PTF coated stainless steel pestle and driven by a dental drill. The homogenate was centrifuged at 12,000g for 20 min at 4°C, according to Saavedra et al. (1973) and the supernatant was transferred in 2 x 10 $\mu$ l aliquots to silanized 2" x  $\frac{3}{8}$ " glass tubes, capped and stored at -70°C until the catecholamines were measured. The remainder (pellet) was saved for protein estimation, to which 200  $\mu$ l 2M NaOH was added, capped, vortex mixed and kept at room temperature until analysis. A set of six 'Reagent Blank' tubes per assay containing 10 $\mu$ l 0.1M HCl/DTPA/ $\text{Na}_2\text{S}_2\text{O}_5$  but without tissue was prepared and great care was taken not to contaminate these and other assay tubes. All samples were assayed within 5 days of collection.

### 2.3 Reagents

(a) Standards: Stock solutions of DA, NA and of A were prepared by dissolving 3.095mg 3-hydroxytyramine hydrochloride, 9.640mg Arternol bitartrate and 4.550mg Epinephrine bitartrate, separately, in 100ml of 0.001M HCl containing 1mM DTPA and 10mM DTT. 200 $\mu$ l of these solutions were aliquoted and stored at -70°C until use. These standard aliquots remained unchanged for three months, the longest time period over which they were used. At the time of assay, they were further diluted in

0.2M  $\text{HClO}_4$  containing 1mM DTPA and 10mM DTT to give 125pg per 10 $\mu$ l. Internal standards were prepared by adding 125pg of each standard to one of the 10 $\mu$ l aliquots of the tissue extract (previously described) and the external standards were prepared by adding 125pg to 10 $\mu$ l 0.1M  $\text{HCl}$ /DTPA/ $\text{Na}_2\text{S}_2\text{O}_5$ .

(b) Buffers:

(i) 2M Tris acetate buffer (pH=8.8)

2M Tris acetate and 2M Tris base were mixed in an approximate ratio of 1:2 to give a final pH of 8.8, adjusted if necessary

(ii) 0.5M Sodium borate buffer (pH=9.0)

0.5M Boric acid and 0.5M Potassium chloride were added to 500ml double distilled water and gently warmed until dissolved. The pH was adjusted to 9.0 by adding 1M NaOH and further diluted to 1.0 litre.

(iii) 0.4M Sodium borate buffer (pH=11.0)

0.4M Boric acid 0.4M Potassium chloride were added and the pH adjusted to 11.0 as described above.

(c) 0.2M  $\text{HClO}_4$ :

This was prepared by diluting 2.139ml of 60-62%  $\text{HClO}_4$  to 100ml with double distilled water.

(c)  $\text{MgCl}_2$  Solution (40mM)

This was made by adding 52mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  in 1.0ml of 0.2M  $\text{HClO}_4$  to give a final concentration of 40mM when added to 5 $\mu$ l in the assay tube.

(e) Phosphotungstic acid solution ['PTA', 1% (w/v)]

1% (w/v) PTA was made by dissolving 1g in 100ml of 0.5M  $\text{HCl}$ .

(f) Dithiothreitol Solution ['DTT', (10mM)]

This was made up fresh when required by adding 15.4mg of DTT per 10ml of 0.2M  $\text{HClO}_4$  containing 1mM DTPA.

(g) Scintillation fluid:

4g of PPO and 50mg of POPOP were added to 1 litre of toluene and to this 25ml of DEHP (2.5%, v/v) was added. The solution was allowed to mix on a mechanical stirrer for >5hr and 5ml per scintillation vial was routinely used.

(h) Homogenizing medium (0.1M HCl/1mM DTPA/0.1% (w/v) Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>):

This was prepared by diluting 2M HCl to 0.1M, to which 1mM diethylene triamine penta acetic acid (39.34mg/100ml) was added. To this stock solution 0.1% (w/v) sodium metabisulphite (1mg/ml) was added freshly when required to use.

(i) Thin-layer chromatographic solvent

This was made from chloroform:methanol: 70% ethylamine::16:3:2 (v/v/v).

2.4 Standard assay procedure

Each assay included:

(i) Six 'reagent blank' tubes;

These were prepared by omitting catecholamines and the tissue extract. This was a modification of Sällar & Zigmond's assay in which 'tissue blanks' were prepared by adding 0.2M HClO<sub>4</sub> containing CaCl<sub>2</sub>·2H<sub>2</sub>O (40mM) in place of MgCl<sub>2</sub> to the 10μl tissue extract in order to inhibit COMT reaction. However, 'reagent blanks' were identical to that of 'tissue blanks' and therefore were routinely used for each assay. Blanks prepared in this way are in agreement with an earlier report of Passon & Peuler (1973) in which noradrenaline and adrenaline were measured by a radioenzymatic assay.

(ii) Unknown samples of tissue extract;

2 x 10μl aliquots of either MPOA or MBH supernatants [described previously] were run in parallel.

(iii) Internal standard tubes;



To one of the 2 x 10 $\mu$ l aliquots of the supernatant, [see the sub-section (2.2) on tissue extraction in this section 'C'] 125pg of each DA, NA and A standard was added. This amount of standard was chosen since it was within the range of the standard curve for DA, NA and A (Fig.7). The standard curve was obtained by preparing a set of tubes containing 10 $\mu$ l of the MBH tissue extract pool (10 MBH punches/ 250 $\mu$ l homogenizing medium) and to some of these tubes various amounts of catecholamines were added. The tissue and tissue containing catecholamines were run in parallel. The addition of standards to the tissue serves two purposes:

(a) it acts as a marker to indicate a satisfactory measurement of the known amount of DA, NA and A in the presence of tissue extract

(b) since the other portion of 2 x 10 $\mu$ l supernatant extract contains the same amount of supernatant but without the addition of DA, NA, A standard, the counts per minute (CPM) obtained were matched with the internal standard tube and subtracted from it [Internal standards + 10 $\mu$ l tissue extract (CPM) - 10 $\mu$ l tissue extract (CPM) = Internal standard (CPM) and the absolute CPM thus obtained corresponding to 125pg of each standard (DA, NA, A) was used in calculating the unknown amount of DA, NA and A.

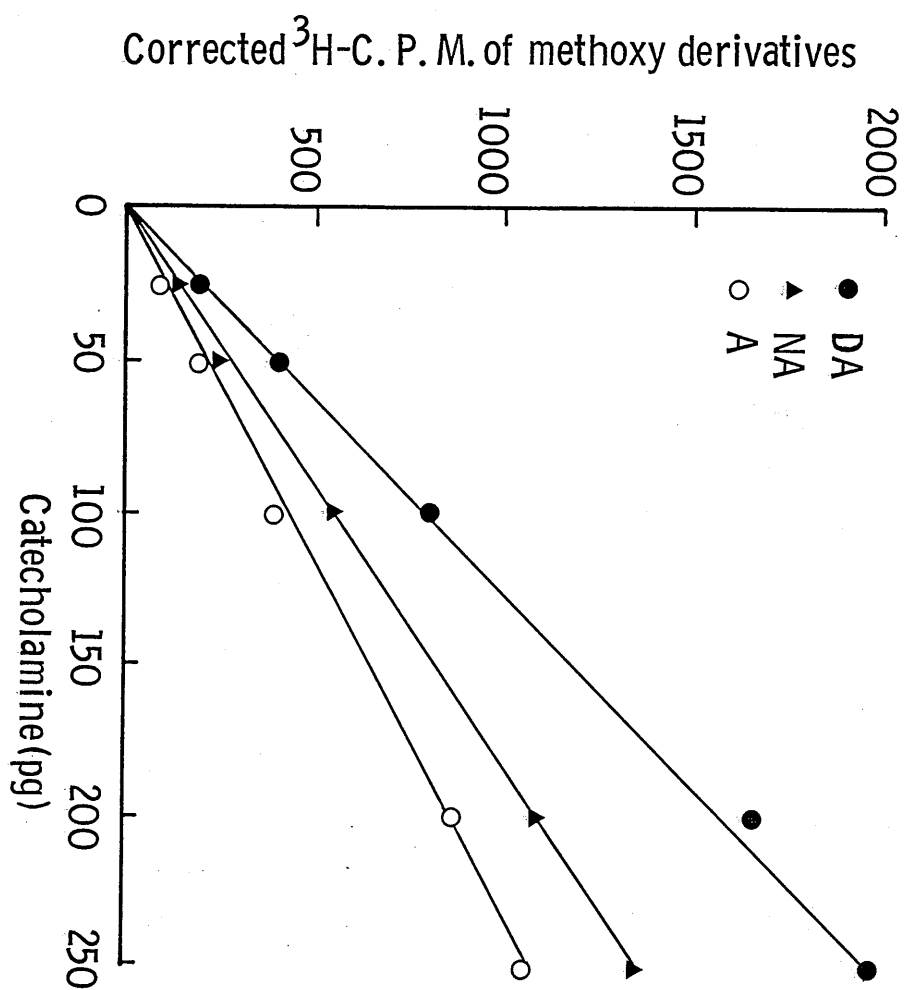
(iv) External standards;

A set of six tubes was prepared by the addition of 10 $\mu$ l 0.1M HCl/1mM DTPA/0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> [homogenizing medium] and 125pg of each DA, NA and A, but devoid of tissue extract. The CPM obtained from these tubes matched very well with the absolute CPM corresponding to DA, NA and A [as described above]. It is assumed therefore, that there was no interference, whatsoever, caused by the tissue extract.

On the day of assay, the following solutions and reagents

Fig.7. Relationship of DA, NA and A concentrations to radioactivity (CPM) as determined by radioenzymatic assay.

Each point is the average of 3 individual results after correction for the 10 $\mu$ l tissue extract.



were made up freshly.

(i) To the stock solution of 0.2M  $\text{HClO}_4$  containing 1mM DTPA, was added DTT (15.4mg per 10ml).

(ii) Preparation of BSA/DTT/Pargyline solution:

The following chemicals were weighed out -

(a) 6mg BSA and 23mg DTT in a plastic tube (PT0944)

(b) 7.8mg pargyline, per ml double distilled water, in a PT0944 tube.

BSA/DTT/Pargyline solution was made by mixing 1.0ml of (b) to (a). Since this solution precipitates out if kept on ice, (b) to (a) was added when needed for the enzymatic reaction mixture. 1 $\mu$ l per each assay tube was added to the reaction mixture. This gave a final concentration of BSA, DTT and Pargyline as 0.18% (w/v), 50mM and 1.25mM respectively [as described by Saller & Zigmond, 1978].

(iii) Preparation of EGTA/Tris-acetate buffer

7.6mg of EGTA was weighed out in a PT0944 tube and to this 1ml Tris-acetate buffer [pH=8.8] was added, corresponding to a final concentration of 5mM EGTA and 500mM Tris-acetate buffer [pH=8.8] when 8 $\mu$ l of this solution was added to the assay tube.

The addition of BSA, DTT, Parglynine and EGTA in the assay tubes prior to incubation is essential since it serves two purposes:

(a) keeps COMT in an active form

(b) minimises the errors involved in the estimation of catecholamines.

Read et al. (1970) found that a large dilution of enzymes can possibly inactivate them. The problem can be minimised by addition of BSA at a final concentration of approx. 0.1-1% (w/v). Similarly, the oxidation of thiol groups of enzymes can cause inactivation (Cleland,

1964). The enzymes are inhibited due to conversion of SH groups into s-s-groups. This problem can be overcome by inclusion of DTT. McCaman et al., (1965) found that the COMT preparation is not entirely pure in that it contains a small amount of monoamine oxidase (MAO) activity. The presence of this enzyme (MAO) in the assay might interfere with O-methylation of catecholamines. However, this problem can be overcome by inclusion of an inhibitor of MAO e.g. Pargyline. Zammit & Newsholme, 1976, found that  $\text{Ca}^{2+}$  concentration can be minimized in a solution by the addition of EGTA which is more specific for  $\text{Ca}^{2+}$  than other chelating agents. It is therefore important to use EGTA when  $\text{Mg}^{2+}$  is essential for the activity of COMT.

(iv) Dilution of standards:

One aliquot of each of the DA, NA and A concentrated standards were thawed and kept on ice. 10 $\mu$ l of each of the DA, NA and A aliquots was added to 9.990ml  $\text{HClO}_4$ /DTPA/DTT solution, mixed briefly and kept on ice until used.

(v) Carrier solution:

This was made by dissolving 1mg per ml of each 3-methoxytyramine, noremetanephine and metanephine in 0.1% (w/v)  $\text{Na}_2\text{S}_2\text{O}_5$  and kept at 4°C until used.

(vi) Reagent I:

was made by mixing equal volumes of 0.2M  $\text{HClO}_4$ /DTPA/DTT and  $\text{MgCl}_2$  solutions corresponding to 5 $\mu$ l of each solution per assay tube and depending on the number of total blanks and tissue tubes to be assayed.

(vii) Reagent II:

was made by mixing equal volumes of 0.2M  $\text{HClO}_4$ /DTPA/DTT containing 125g of DA, NA, A and  $\text{MgCl}_2$  solution, corresponding to 5 $\mu$ l

of each solution per assay tube and depending on the total number of internal and external standard tubes.

(viii) Preparation of the enzymatic Reaction mixture:

This was made by mixing 8 $\mu$ l EGTA/tris-acetate buffer, 1 $\mu$ l BSA/DTT/Pargyline, 4.5 $\mu$ l COMT (optimum volume) and 1.5 $\mu$ l of S-adenosyl [methyl-<sup>3</sup>H] methionine [1.5 $\mu$ Ci]; per assay tube. All the above amounts were multiplied by the number of total tubes and then added in that proportion, mixed gently (avoiding frothing) and kept on ice until used.

A. All the assay tubes were kept on ice -

(a) 10 $\mu$ l of reagent I was added to each of the 'Reagent blank' and tissue tubes.

(b) 10 $\mu$ l of Reagent II was added to internal and external standard tubes.

B. 15 $\mu$ l of the enzymatic reaction mixture was added to each tube and immediately vortex-mixed briefly. The addition of reagents and enzymatic mixture to all tubes was done within 10 min. to ensure that all the components of reaction remained stable (see Saller & Zigmond, 1978).

C. The reaction was initiated by placing these tubes into a shaking water bath at 30°C.

D. The reaction was terminated after 30 min. by placing them in an ice bath and adding 250 $\mu$ l 1% PTA (preparation described) and briefly vortex-mixed.

E. These were centrifuged on Mistral 6L at 15,00g for 30 min. at 10°C. Although Sallar & Zigmond (1978) used 10 $\mu$ l 20% (w/v) PTA, in the present method 250 $\mu$ l of 1% PTA was used since a preliminary study

showed that this amount of PTA in 0.5M HCl when added to the tubes gave a much better sample to blank ratio.

F. 200 $\mu$ l of the supernatant was transferred to silanized round-bottomed 10 x 1.5cm glass tubes containing 1ml 0.4M borate buffer (pH=11): carrier solution (20:1, v/v). 3ml of toluene/isoamyl alcohol (3:2, v/v) was then added to these tubes, vortex-mixed for 30 seconds and centrifuged at 15,00g for 10 min. at 10°C.

G. 2.4ml of the organic phase was transferred to silanized conical pointed glass tubes (10 x 1.5cm) containing 50 $\mu$ l 0.1M HCl/1mM DTPA/0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> vortex-mixed for 30s and centrifuged at 15,00g for 10 min. at 10°C.

H. The organic phase was aspirated and 40 $\mu$ l of the acid phase was transferred to LP2 tube. This was either applied to the TLC plates or capped and stored overnight at -20°C until required. This method of toluene/isoamyl alcohol extraction followed by re-extraction into 0.1M HCl is in accordance with Cuello's (1979) method, however, some minor modifications were made. It was found that the method of solvent extraction was necessary to reduce the radioactivity of the blank values and thus, to increase the sensitivity of the assay. This has been used throughout the present study.

## 2.5 Thin-layer Chromatography:

LK6DF TLC plates were sprayed with an antioxidant, 0.1% (w/v) DTT solution in absolute alcohol and left in a fume cupboard for 30 min. at room temperature for drying. 5 $\mu$ l of the carrier solution (see V of the standard assay procedure of this chapter) was applied to each channel of the TLC plate with a Hamilton syringe. This was followed by addition of 30 $\mu$ l of the acid phase (see 'H' of the standard assay procedure) to the same channels. A set of 'reagent blank' matching 'tissue' and 'internal standards', and external standards were applied to the same

TLC plate in order to avoid any possible variation involved in the inter-TLC plate developing procedure. The plates were dried in a fume cupboard for 30min. at room temperature and then developed for 90 min. in a chromatographic tank containing chloroform/methanol/ethylamine (16:3:2) in the dark. The developed plate was then dried in a fume cupboard for 10 min. at room temperature and the 'spots' corresponding to methoxytyramine (MT), normetanephrine (MN) and metanephrine (M) were visualized under U.V. light (254 nm). A preliminary study was performed in which authentic MT, MN and M were applied to TLC plates and this confirmed the separation and location of the spots which is in agreement with the R<sub>f</sub> values [the distance travelled by a methoxyderivative spot divided by the distance travelled by the solvent front for MT, NM and M, as reported by Saller and Zigmond (1978)]. The areas corresponding to MT, NM and M were scraped off the plate and transferred to scintillation vial inserts containing 0.1ml borate buffer (pH=10). 5ml of scintillation fluid containing 2.5% (v/v) DEHP was subsequently added and vortex-mixed for 30s. The inserts were put into scintillation vials and counted on a Packard  $\beta$ -counter for 10 min. each after keeping the vials at room temperature for >1hr. Although Saller and Zigmond have reported the use of 5ml of Econofluor [pre-mixed scintillation fluid], however, the performance characteristics of the present toluene base scintillant [preparation described] are similar to Econofluor [NEN Chemicals, U.S.A]. This is reflected by the similar tritium efficiencies (i.e. Econofluor efficiency, approx. 56%; present scintillation fluid, approx. 53% (Table 1).

**2.6 Counting Efficiency.** The counting efficiency of Packard  $\beta$ -counter for tritium was determined by the 'internal standard method'. In this method 25 $\mu$ l of [<sup>3</sup>H]-toluene containing 500 disintegration per minute ( $\Delta$ DPM) were added to the various samples of MT, NM and M obtained after



radioenzymatic and TLC steps as described in this chapter. These were recounted and the counting efficiency was calculated as shown below:-  
 A sample [MT, NM, M] of unknown disintegration per minute (DPM) was counted on the  $\beta$ -counter and this gave a certain number of counts per minute (CPM 'C') [e.g. metanephrine (M) CPM(C) = 200]. This was then recounted (total CPM = C +  $\Delta$ C or  $\Delta$ C = total CPM - C; total CPM = 450 and  $\Delta$ C = 250) after the addition of  $\Delta$ DPM and therefore 250 CPM ( $\Delta$ C) corresponds to the added  $\Delta$ DPM. The counting efficiency (E) (%) =  $\frac{\Delta C}{\Delta DPM} \times 100$

$$\therefore E = \frac{250}{500} \times 100 = 50\%$$

The efficiency was consistent from sample to sample as indicated by low S.E.M. (Table 1). This was expected because all the samples were of the same composition with respect to any quenching agent present. This method of internal standardization was adopted initially, but its continuous use was not feasible. Instead the 'external standard channel ratio' method was routinely used. The ratio of counts obtained in two separate high and low energy channels in response to gamma-rays emitted by a radiation source [ $^{137}\text{Cs}$ ] external to the counting vial but contained within the instrument was related to the [ $^3\text{H}$ ]-efficiency of 53% [calculated by the 'internal standard' method]. This method is especially useful if the activity of the sample is too low to use the 'channel ratio' method. Consistent external standard channel ratio values were obtained and therefore counting efficiency does not vary from sample to sample. Table No.1. Counting efficiency (E) of tritium ( $^3\text{H}$ ) added to the final liquid scintillation mixture

Fraction	$E(\%) = \frac{\Delta C}{\Delta DPM} \times 100$ (Mean $\pm$ S.E.M., n = 5)
Methoxytyramine	53.4 $\pm$ 2.6
Normetanephrine	53.0 $\pm$ 1.3
Metanephrine	51.5 $\pm$ 2.0

## 2.7 Calculations:

DA, NA & A concentrations present in tissue specimens were determined using a pre-programmed computer [Apple II]. The determination was performed by evaluating radioactivity (CPM) present in the 'reagent blank', 'tissue' and 'internal standard' samples. Internal standard and unknown samples ('tissue') were then corrected for 'tissue' (CPM) and 'reagent blanks' respectively. Corrected CPM for unknowns were compared to those of 125pg pure standards. However, 'A' CPM were further corrected for cross-contamination of DA (0.7%) and NA (0.46%) (see Saller & Zigmond, 1978) before comparison to those of 125pg 'A' standard. Since linearity of the standard curve (Fig.7) was proven for at least up to 250pg which lies well within the range of the maximum levels measured in any sample, quantification could be made by means of a calibration factor (absolute CPM for standards).

## 2.8 Reliability Criteria:

Precision is estimated from the coefficients of variation as discussed in the section 'A' of this chapter.

(a) Inter-assay coefficient of variation for adrenaline:

<u>Mean</u> (CPM)	<u>Standard deviation</u>	<u>Coefficient of</u> <u>variation</u>
1221	127.66	10.45%
n = 7		

(b) Intra-assay coefficient of variation:

<u>Mean</u> (CPM)	<u>Standard deviation</u>	<u>Coefficient of</u> <u>variation</u>
1283	48.40	3.77%
n = 10		

## 2.9 Sensitivity & Specificity:

The sensitivity (2 x S.D. of the reagent blanks) resulted in

DA = 8.0pg, NA = 11.5, A = 7.5pg

These were considered to be the smallest amount of catecholamines which could be reliably measured and values lower than these were designated non-detectable (ND). Although the sensitivity of the assay can be increased by using [ $^3\text{H}$ ]-SAM of much higher specific activity. Nevertheless, SAM (15Ci/mmol) was found to be both adequate and economical since the sensitivities obtained were consistent with reliable measurements of catecholamine concentration in discrete areas of the brain.

This assay is considered to be highly specific for each of the catecholamines. This is due to its specificity derived at three different steps:

(a) the enzyme, COMT is specific for catechols regardless of substituent on the aromatic ring, however, monophenols are not methylated by COMT (Axelrod and Tomchick, 1958).

(b) the extraction of COMT products into toluene-isoamyl alcohol (see Fig. 6 in this chapter) from borate buffer (pH 10; final pH of the mixture) occurs most readily with o-methylated catechols but not with catechols (Axelrod, 1962).

(c) the TLC step appears to separate o-methylated catechols on the basis of polarity of their side chains. Although specificity of the assay can be assumed to be excellent due to enzymatic labelling with subsequent TLC separation, however there was little cross contamination between the catecholamines. It was found that 0.70% DA CPM and 0.46% NA CPM - cross contaminated - A CPM, therefore corrections were made, by subtracting 0.70% DA CPM and 0.46% NA CPM from 'A' CPM, in the calculations.

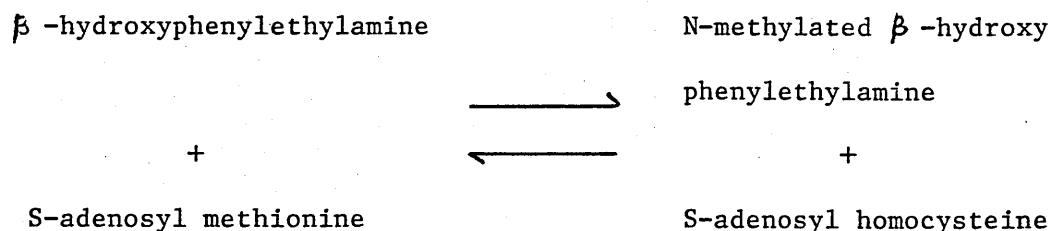
## SECTION 'D'

### RADIOMETRIC ASSAY OF PHENYLETHANOLAMINE-N-METHYL TRANSFERASE

#### 1. Introduction

The conversion of noradrenaline (NA) to adrenaline (A) is the final step in catecholamine biosynthesis (Blaschko, 1939). Phenylethanolamine-N-methyl transferase (PNMT), EC2.1.1.28) is the enzyme which catalyzes this reaction. The reaction involves N-methylation of NA to A in the presence of S-adenosyl methionine (SAM) as the methyl donor.

The enzyme was first reported by Kirshner and Goodall in 1957, to be present in the soluble fraction of homogenates of bovine adrenal medulla. The enzyme is also known more conveniently as noradrenaline-N-methyl transferase (NMT; Fuller & Hemrich-Luecke, 1983). However, the enzyme exhibits only slight specificity for the substituents attached to the aromatic ring, but a hydroxyl group at the  $\beta$ -position of the side chain of a variety of phenylethylamines is an essential requirement (Marki et al., 1962). In the present work the commonest name, phenylethanolamine-N-methyl transferase (PNMT), was adopted and in general the reaction catalyzed is as follows:



A great deal of work has been done on adrenal PNMT, in order to establish the enzyme properties. Thus Axelrod (1962) first reported the purification and properties of PNMT isolated from monkey adrenals, and showed

that maximum activity occurred from pH 7.5 to 8.2 in 0.1M phosphate buffer and 8.0 to 9.0 in 0.1M tris buffer using normetanephrine as a substrate and SAM the methyl donor. The Michaelis-Menten constant [defined as the substrate concentration at which enzyme velocity is half maximal] for N-methylation of normetanephrine was found to be  $5 \times 10^{-15}$  M. Furthermore, this author discovered that the enzyme activity was almost completely inhibited with p-chloromercuribenzoate indicating the presence of essential sulfhydryl groups. With respect to substrate specificity, it was found that the  $\beta$ -hydroxylated phenylethylamines and their derivatives are methylated, but not phenylethylamine. Connet and Kirshner (1970), studied the purification and properties of bovine adrenal PNMT and have shown that the enzyme contains sulfhydryls which is in agreement with that of Axelrod (1962). However, the optimum pH in both 0.1M phosphate and borate buffers is very precise, 7.9 in contrast to Axelrod's results. The molecular weight determined by these authors as assessed from the calibration curve established by the elution of marker proteins, for PNMT (mono-meric protein) is 38,000 which is identical to that obtained for rat adrenals (Yu, 1978). However, the optimum pH is different from that of Yu's results, since this author found it to be 9.0 in either tris or phosphate buffer. Species difference might be the reason for differences in the pH optimum. Furthermore, Connet & Kirshner found the presence of only one form of PNMT in bovine adrenals and the absence of isoenzymes; the enzyme was found to be stable for at least 50min. at 39°C, but rapidly destroyed at 45°C.

#### Brain phenylethanolamine-N-methyl transferase

Although phenylethanolamine-N-methyl transferase was originally identified in adrenal medulla, a similar enzyme in low concentration has been found in the mammalian central nervous system (CNS). Thus, Axelrod (1962) indicated that minute amounts are present in the midbrain area

of the rabbit. McGeer and McGeer (1964) also indicated its presence by observing that a small fraction of [ $^3\text{H}$ ]-noradrenaline injected directly into monkey or cat brainstem and caudate could be recovered as [ $^3\text{H}$ ]-adrenaline. In the past years, its presence in mammalian CNS has been well established (Ciaranello et al., 1969; Pohorecky et al., 1969; Hökfelt et al., 1973; Saavedra et al., 1974b; Chalmer et al., 1981 and Fuller et al., 1982) and Yu (1978) has reported its comparison with adrenal PNMT. According to Yu (1978), PNMT isolated from the rat brain possesses some physiochemical properties which are different from that of PNMT isolated from the adrenal medulla. The differences are:

(1) the brain PNMT molecular weight is 25,000 whereas the adrenal PNMT 38,000 identical to that obtained for bovine adrenals (Connett and Kirshner, 1970); (2) the pH optima in phosphate and tris buffers was found to be 9.0 and 7.8 for the partially purified rat adrenal and brain PNMT respectively; (3) the Michaelis-Menten constant ( $K_m$ ) for  $\beta$ -hydroxylated phenylethylamines indicated that the brain PNMT was higher than that of adrenal PNMT. Perhaps the low ratio of adrenaline to noradrenaline (1:10) [Mefford et al., 1977; Koslow and Schlumpf 1974; Gunne, 1962; Vogt, 1954] is a consequence of the low activity of the brain PNMT due to high  $K_m$  value. The optimum pH of 7.9 for brain PNMT is in agreement with the results of Ciaranello et al., (1969) and Pendleton et al., (1977), but different from that of Saavedra et al., (1974b) who found it to be 8.6.

#### Non-specific N-methylation in the brain

In addition to PNMT (soluble protein, present in the cytosol) there is another soluble protein or family of proteins present in the cytosol known as non-specific N-methyl transferase. The presence of this protein in the brain was reported by Saavedra et al., (1973). This enzyme N-methylates a large number of substrates, tryptamine,  $\beta$ -hydroxylated

phenylethylamine, phenylethylamine etc., and exhibits pH of 7.9, similar to brain PNMT (Pendleton et al., 1977). The non-specific activity was found to be highest in the cerebral cortex and striatum and lowest in the medulla-pons and olfactory lobe of the rat brain. Similarly, Pendleton et al., (1977) have reported a regional distribution of non-specific N-methyl transferase activity. The spinal cord, brain stem and cerebellum had negligible activities, but significantly higher in the midbrain and forebrain of the rat.

In the light of the above survey of previous work on pheylethanolamine-N-methyl transferase in the brain and the adrenal medulla, it is clear that discrepancies exist with regard to the optimum pH. Since the main objective was to establish a highly sensitive and specific assay so that PNMT activity present in the discrete areas of the hypothalamus could be measured, the characteristics of PNMT were studied in detail.

## 2. PNMT Assay

A review of the literature concerning PNMT revealed that various authors have measured PNMT activity by a basic radioisotopic method with certain modifications (Axelrod, 1962; Pohorecky et al., 1969; Connet and Kirshner, 1970; Saavedra et al., 1974b; Pendleton et al., 1977; Howe et al., 1981; Fuller et al., 1982). In the present study PNMT activity was measured by a modification of the method previously described by Pendleton et al., (1977). The basic principle of this assay is shown in Fig.8.

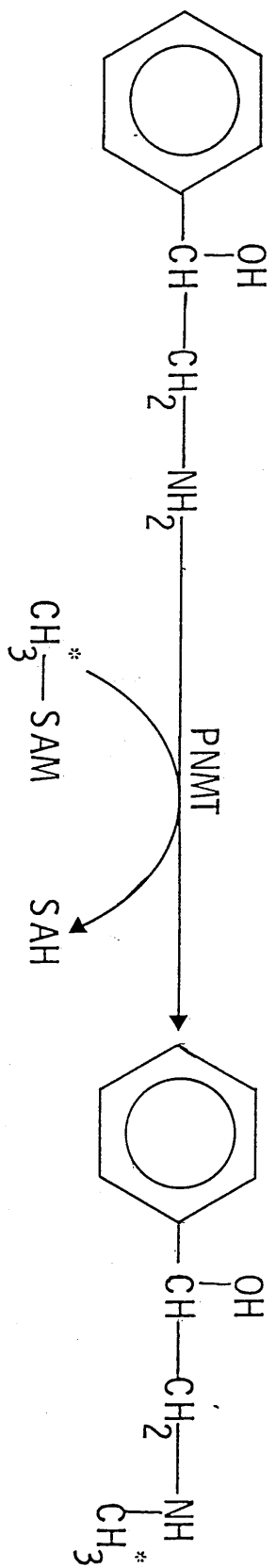
## 3. Materials and Methods

Details of the suppliers for most of the reagents used in this assay are described in the section on radioenzymatic assay of catecholamines, however, the remainder are mentioned here.

Fig.8. Diagrammatic representation of the basic principle of phenylethanolamine-N-methyl transferase (PNMT) assay, methods of tissue preparation, and estimation of PNMT activity.

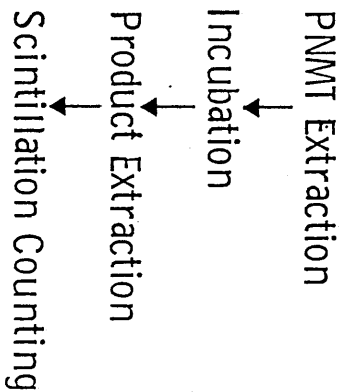


RADIOMETRIC ASSAY FOR PHENYLETHANOLAMINE - N - METHYL TRANSFERASE (PNMT)



Incubation 60 mins

Tissue homogenized in isotonic KCl  
containing 0.5 mM dithiothreitol



Intra - Assay Coefficient of variation: 7.8%  
Inter - Assay Coefficient of variation: 13 %  
Sensitivity of the assay: 1 P mol / hr / mg Protein

### 3.1 Reagents

(a) Homogenizing medium: 1.19% (w/v) KCl containing 0.5mM DTT,  
1.19% KCl - 1.19g/100ml double distilled water

0.5mM DTT - 0.385mg DTT [made fresh] 4mg DTT was  
dissolved in 500 $\mu$ l 1.19% KCl - 48 $\mu$ l of this solution was diluted in  
5ml with 1.19% KCl.

Pendleton et al., (1977) homogenized brain tissue in 1.5 volumes (w/v)  
of isotonic (1.19%) KCl without DTT.

Since a survey of the literature indicated that PNMT contains sulfhydryl  
groups, which are essential for maximum activity, a recommended amount  
of DTT was also included in the homogenizing medium and 10 volumes  
instead of 1.5 volumes was found to be more convenient for homogenizing  
brain tissue.

#### (b) Buffers:

Tris-HCl buffer, 0.2M, pH= 8.8

(1) 0.4 M HCl

(2) 0.4 M tris base

(1) was added to (2) until pH reached 8.8 and then diluted  
to 200mls with double distilled water.

Sodium borate buffer, 0.5M, pH = 10

30.92g boric acid and 37.28g potassium chloride were added  
to 500mls double distilled water (slight warming is necessary), then  
added 1M NaOH until pH reached 10 and then diluted it to 1 litre.

#### (c) $\beta$ -Hydroxyphenylethylamine solution (BHPEA, 20mM)

[13.78mg/5ml double distilled water]

The stock solution of BHPEA kept well at 4°C for at least  
two weeks [the maximum time for which one preparation of the stock  
solution was routinely used]. BHPEA was supplied by Sigma Chemical  
Company, U.K.

(d) Phenylethylamine and tryptamine solutions

Each of these solutions was prepared to give a final concentration at  $1.6 \times 10^{-3} \text{ M}$  in the incubation medium which is similar to that described by Saavedra et al., (1973).

(e) Toluene-isoamyl alcohol mixture (97:3, v/v)

(f) 0.1M HCl was kept as stock solution at 4°C for several months

(g) Scintillation fluid (ACS II)

Aqueous containing scintillation fluid supplied by Amersham International was routinely used and gave an efficiency of 50% for tritium [Amersham International personal communication].

### 3.2 Tissue extraction

(a) Characteristics of PNMT were studied using the whole hypothalamic crude extract of phenylethanolamine-N-methyl transferase and this was prepared by homogenizing 4 - 8 hypothalami.

Albino Wistar male adult rats were stunned and rapidly decapitated with large scissors. The brain was removed immediately and placed ventral side uppermost on ice-cooled metal plate. The hypothalamus (an area of the diencephalon, lying at the base of the brain, ventral to the thalamus, and forming the floor and part of the lateral walls of the third ventricle) was dissected along neuroanatomic boundaries. A coronal cut was made rostrally through the optic chiasma and caudally through the mammillary bodies and then laterally through each lateral ventricles. The isolated hypothalamus was, immediately transferred to a metal plate on solid CO<sub>2</sub> (dry ice). The hypothalami were weighed quickly using a micro-balance [Mettler, AE163, Mettler Instrument Switzerland] and then homogenized in ice-cold 10vol (w/v) (Saavedra et al., 1974b) homogenizing medium (1.19% KCl/0.5M DTT), (Pendleton et al., (1977)). The homogenate was transferred to ice-cold centrifuge tubes and spun at 20,000g for 30min at 4°C (Fig.8). The supernatant

was transferred to silanized glass tubes, capped and kept at  $-70^{\circ}\text{C}$  until assayed. To the remaining pellet 2M NaOH [200 $\mu\text{l}$  per mg wet weight of the hypothalamus] was added, mixed, and then capped and left at room temperature until assayed for protein content.

(b) The MPOA and MBH samples

Samples from the MPOA and MBH were obtained as described previously (see Section 'B' in this chapter). They were homogenized in 60 $\mu\text{l}$  homogenizing medium and then transferred to ice-cold LP2 tubes. The tubes were spun at 20,000g. The supernatant was transferred in 2 x 25 $\mu\text{l}$  aliquots to silanized 3" x  $\frac{1}{2}$ " glass tubes, capped and kept at  $-70^{\circ}\text{C}$  until assayed. 200 $\mu\text{l}$  2M NaOH was added to the remaining pellet and the tubes were mixed briefly, capped and kept at room temperature before being assayed for their protein content (see Appendix for the protein assay details).

3.3 Characteristics of pheylethanolamine-N-methyl transferase:

It is well established that a variation of enzyme activity takes place as a result of a change in temperature (see for example Alp et al., 1976; Arch and Newsholme, 1976). However, the effect of temperature on PNMT activity was not explored in the present studies since the preferred temperature ( $37^{\circ}\text{C}$ ) at which physiologically meaningful results are to be obtained, has been frequently used by other authors who have reported measurements of PNMT activity (Saavedra et al., 1974b; Pendleton et al., 1977; Yu, 1978).

(a) Effect of pH on the specific activity of PNMT [N-methylation of  $\beta$ -hydroxyphenylethylamine (BHPEA), pmol/h/mg]:

50 $\mu\text{l}$  of the rat hypothalamic supernatant (the preparation of which has already been described in section 3.2 of this chapter) was incubated at  $37^{\circ}\text{C}$  for 60min with 80 $\mu\text{l}$  BHPEA, 0.54 $\mu\text{mol}$  of [ $^3\text{H}$ ]-methyl-S-adenosyl methionine (specific activity, 15Ci/mmol) (see Saavedra et al., 1974b;

Pendleton et al., 1977) and 5 $\mu$ mol of tris buffer at varying pH (7.4, 7.9, 8.4, 8.9 and 9.2) in a final volume of 150 $\mu$ l. PNMT specific activity was plotted against pH and a bell-shaped curve was obtained and the optimum pH being 8.85 as shown by the arrow (Fig.9). This contrasts with studies by Pendleton et al., (1977) in which phosphate buffer of pH7.9 was used in order to measure rat brain PNMT activity. However, the optimum pH obtained in the present study was commensurate with that pH used by Saavedra et al., (1974b) for the measurement of PNMT activity in rat brain nuclei.

(b) Effect of concentration of BHPEA on the specific activity of PNMT (N-methylation of BHPEA; pmol/h/mg):

50 $\mu$ l of rat hypothalamic supernatant was incubated at 37°C for 60min with 80 $\mu$ l of a solution containing 5 $\mu$ mol of tris buffer (pH 8.8), 0.54 nmol of [<sup>3</sup>H]-methyl-s-adenosyl methionine and 20 $\mu$ l of a solution containing varying amounts of BHPEA (45, 120, 195, 255 and 360 nmol) in a final volume of 150 $\mu$ l. The amount of BHPEA required in order to get an optimum PNMT activity is shown by the arrow in the hyperbolic curve (Fig.10; BHPEA concn.240nmol/150 $\mu$ l or 1.60mM). This amount of BHPEA required to obtain optimum conditions is in agreement with data previously published by Pendleton et al., (1977).

(c) Effect of [<sup>3</sup>H]-methyl-s-adenosyl methionine on the specific activity of PNMT:

50 $\mu$ l of rat hypothalamic supernatant was incubated at 37°C for 60min with a solution of 100 $\mu$ l containing 5 $\mu$ mol tris buffer (pH 8.8), 240nmol of BHPEA and various amounts (37.5, 75, 150, 300, 450, and 600 pmol) of [<sup>3</sup>H]-methyl-s-adenosyl methionine. The volume changes caused by the addition of [<sup>3</sup>H]-SAM were negligible. The optimum concentrations of SAM required to obtain maximum PNMT activity is shown by the arrow (Fig.11; optimum concentration of SAM, 450pmol/150 $\mu$ l or 3 $\mu$ M). This

Fig.9. Effect of varying pH on the specific activity of phenylethanolamine-N-methyl transferase (PNMT). The standard assay pH (8.85) is indicated by the arrow.

Each point is a mean of 3 determinations.

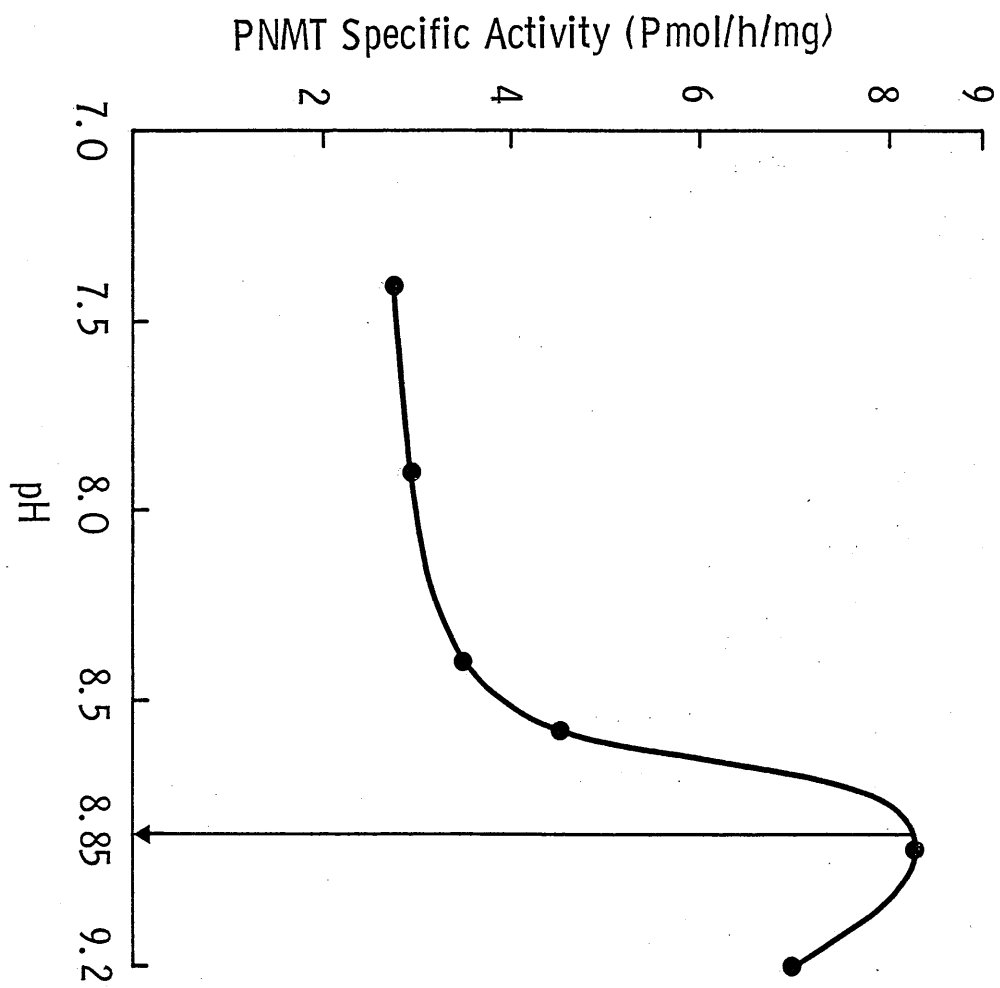


Fig.10. Effect of varying the concentration of  $\beta$ -hydroxyphenylethylamine (BHPEA) on the specific activity of phenylethanolamine-N-methyl transferase (PNMT). The standard assay concentration of total BHPEA (1.60mM) is indicated by the arrow.

Each point is a mean of 3 determinations.



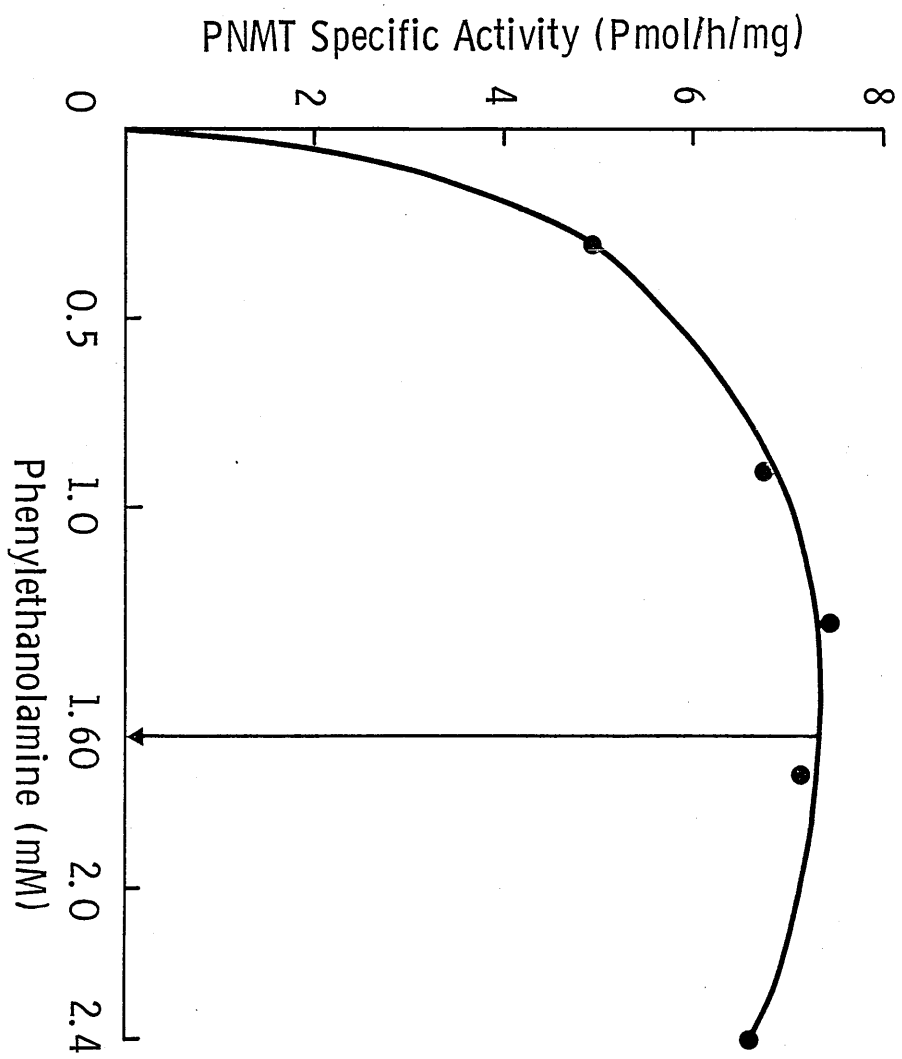
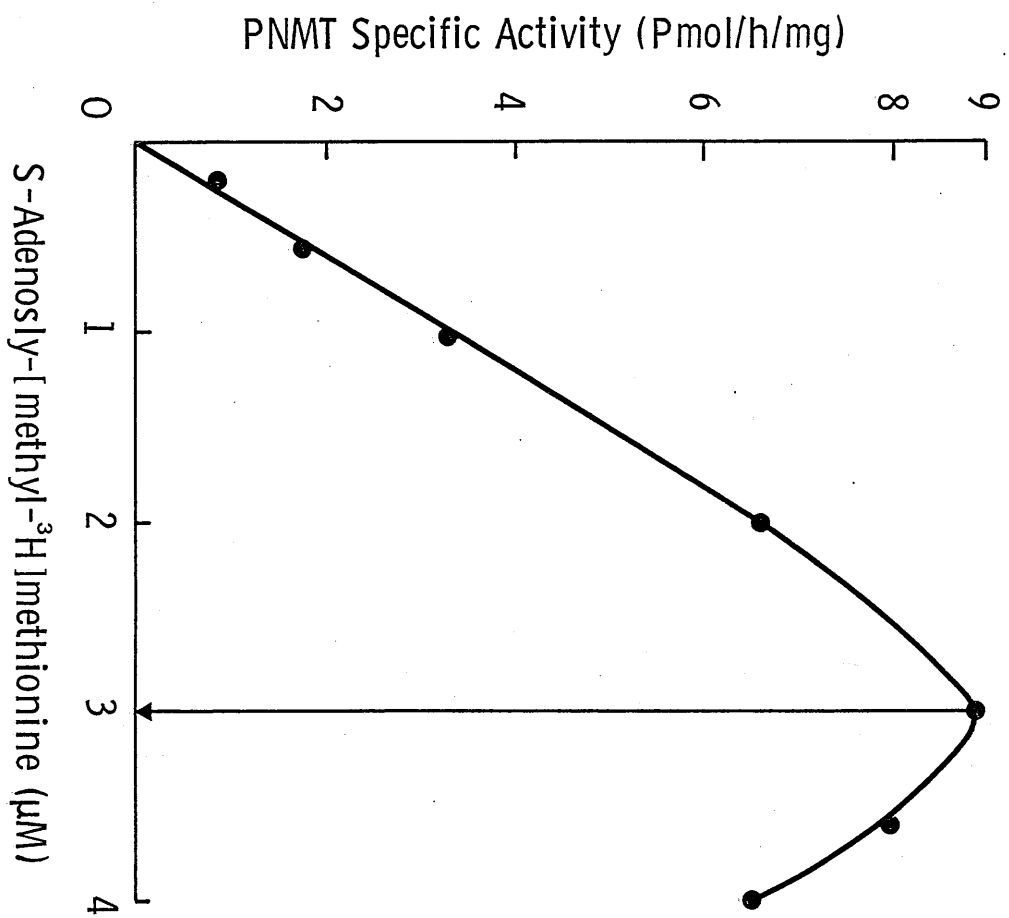


Fig.11. Effect of varying the concentration of S-adenosyl-(methyl-<sup>3</sup>H) methionine (SAM) on the specific activity of phenylethanolamine-N-methyl transferase (PNMT). The standard assay concentration of total SAM (3 $\mu$ m) is indicated by the arrow.

Each point is a mean of 2 determinations.






concentration is similar to that reported by Saavedra et al., (1974b) since they used 3.5 $\mu$ M concentration of [<sup>3</sup>H]-SAM. However, labelled SAM supplied by Amersham International had a specific activity of 15 Ci/mmol whereas Saavedra et al (1974b) used 4.5 Ci/mmol of labelled SAM in order to measure PNMT activity in brain nuclei. Consequently, it was worthwhile making an attempt to cut down the amount of labelled SAM and replace it with an equal amount of unlabelled SAM. This worked well and subsequently a mixture of labelled and unlabelled SAM were used.

(d) Specific and non-specific PNMT activity:

50 $\mu$ l of rat hypothalamic supernatant was incubated at 37°C for 60min with a 100 $\mu$ l solution containing 5 $\mu$ mol of tris buffer (pH 8.8), either 240 nmol of BHPEA and 240 nmol of tryptamine or 240 nmol of pheylethylamine (PEA) and 450 pmol of SAM (225pmol of labelled SAM and 225 pmol of unlabelled SAM). The histograms of specific and non-specific activity indicate that a negligible amount of non-specific activity was present when measured by this assay (Fig.12). This suggests that the present radiometric assay is highly specific. However, Pendleton et al., (1977), have reported much higher non-specific activities in a combined section of rat brain mesencephalon and diencephalon. The difference might be accounted for by the non-specific activity present in the mesencephalon or due to the different pH of the enzymatic reaction mixture since they used a buffer of pH 7.9. It is possible that non-specific methyl transferase is more active at pH 7.9 than 8.8.

(e) Effect of time of incubation on the PNMT reaction product  
(Pmol/mg)

50 $\mu$ l of the rat hypothalamic supernatant was incubated at 37°C for various times (15, 30, 45, 60 and 75min) with a 100 $\mu$ l solution containing 5 $\mu$ mol tris buffer (pH 8.8), 240nmol of BHPEA and 450 pmol labelled

Fig.12. Specific and non-specific activities present in the pooled rat hypothalamic supernatant. Portions of the supernatant were incubated with either phenylethanolamine (  ) phenylethylamine (  ) or tryptamine (  ) as described in the text.

Each bar represents mean  $\pm$  S.E.M. of 4 separate pooled hypothalamic supernatants.

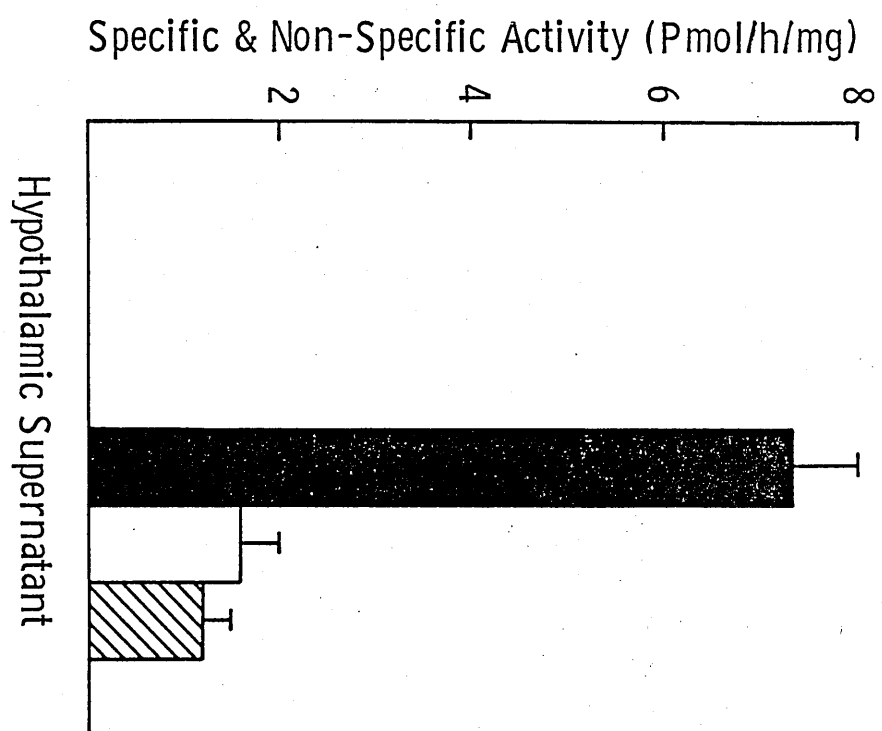
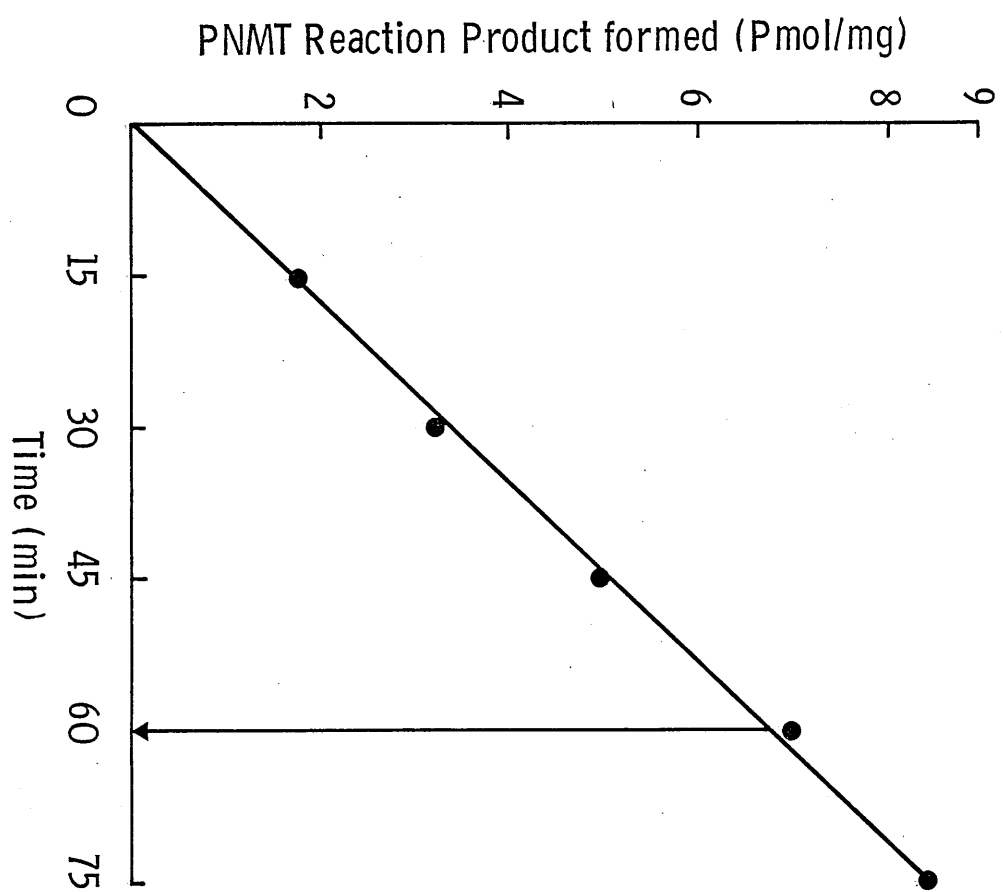


Fig.13. Effect of varying the incubation time on the formation of N-methyl phenylethanolamine. The standard assay incubation time is indicated by the arrow.

Each point is a mean of 3 determinations.





and unlabelled SAM. The plot of the PNMT reaction product versus time of incubation gave a linear curve (Fig.13). One hour of incubation was found to be optimum for PNMT assay conditions. This is in agreement with the previous studies by both Saavedra et al., (1974b) and Pendleton et al., (1977).

(f) Effect of varying the volume of hypothalamic supernatant on the PNMT activity:

100 $\mu$ l of a solution containing 5 $\mu$ mol tris buffer (pH 8.8), 240nmol BHPEA and 225pmol of each labelled and unlabelled SAM was incubated with various amounts of rat hypothalamic supernatant (2.5, 5, 10, 20, 30 and 50 $\mu$ l) in a final volume of 150 $\mu$ l.

Since the range of protein present in the brain punches for which this assay was ultimately used (protein range shown by the hatched area, Fig.14) lies well within the linear curve of the relationship between PNMT activity and the hypothalamic supernatant. Therefore, this observation justifies the validity of this assay.

### 3.4 Standard assay procedure

Each assay included three sets of tubes:

(a) 'Tissue blanks'; were prepared by adding pheylethylamine in place of  $\beta$ -hydroxypheylethylamine (Phenylethanolamine) to one of the 2 x 25 $\mu$ l aliquots of the PNMT extract. However, it was found that exactly the same blank values were obtained by omitting phenylethylamine. Therefore, subsequent blanks were prepared by adding all the reagents in the same proportion except for  $\beta$ -hydroxylphenylethylamine.

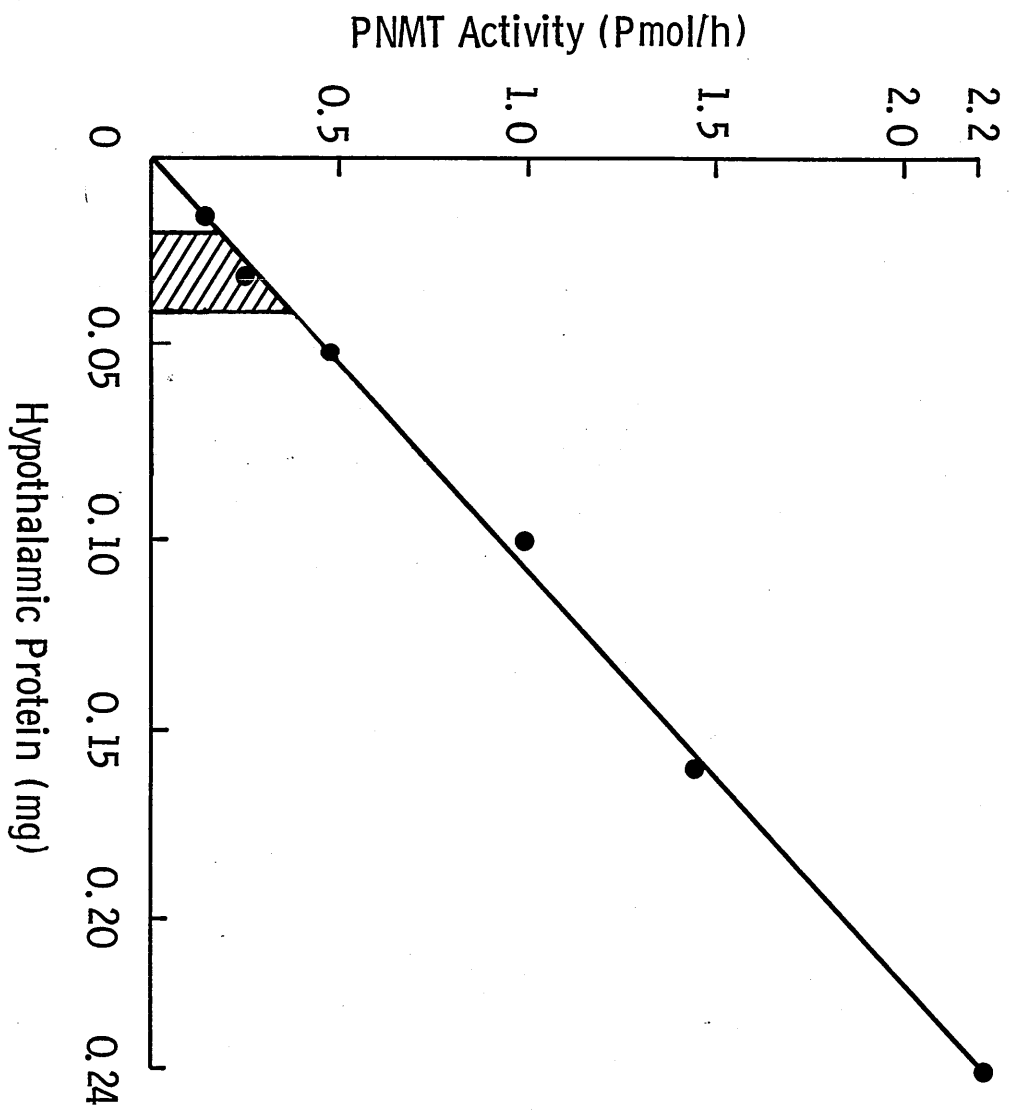
(b) 'Unknown samples'; to the other portion (2 x 25 $\mu$ l aliquots), all reagents and  $\beta$ -hydroxyphenylethylamine were added and run within the same assay including 'tissue blank'.

(c) 'External standard tubes'; a set of eight tubes was prepared by the addition of all the reagents in the unknown samples and in the

Fig.14. Effect of varying the hypothalamic supernatant (which contains protein) on phenylethanolamine-N-methyl transferase (PNMT) activity.

The protein range (0.02 - 0.04mg) in the supernatant of the standard assay for brain punches is indicated by the hatched area.

Each point is a mean of 3 determinations.



same proportion. However, the omission of the tissue extract and the inclusion of two separate standards prepared by redissolving the crystalline bovine PNMT [Sigma Chemical Co., U.K.] in tris buffer (PH = 8.8) was made. These two standards, low and high [four per set] were of the same range as the unknown samples. The consistency of these standards from assay to assay gave a reliable indication that the assay was working satisfactorily.

On the day of assay:

(a) 10 $\mu$ l of BHPEA (0.2 $\mu$ mol) was added to unknowns and external standard samples and 10 $\mu$ l double distilled water to the tissue blanks in place of BHPEA.

(b) An aliquot of the reaction mixture containing 20 $\mu$ l of 0.2M tris buffer (pH = 8.8), 4 $\mu$ l BSA (2%, w/v), 4 $\mu$ l DTT (0.3% w/v), 0.42 $\mu$ l (185pmol) of cold SAM [Sigma Chemical Co., U.K.], 2.92 $\mu$ l (193pmoles) of hot SAM (specific activity, 15Ci/mmol, Amersham International) was added to 'tissue blanks', 'unknowns' and to external standards in a final volume of 125 $\mu$ l. All the three sets of tubes were incubated for 1h at 37°C.

(c) The reaction was stopped after 1h by placing the tubes in ice bath and adding 500 $\mu$ l borate buffer (pH = 10) and vortex-mixed briefly. Then 2ml toluene/isoamyl alcohol was added and vortex-mixed for 30s.

(d) These were centrifuged at 15,00g at 10°C for 10min.

(e) 1.6ml of the organic phase was transferred to 3" x  $\frac{1}{2}$ " silanized glass tubes containing 1ml of 0.1M HCl containing Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (0.1% w/v) and vortex-mixed for 30s.

(f) These were again centrifuged at 15,00g for 10min and the organic phase aspirated. From the aqueous phase 0.8ml was transferred to scintillation glass vials containing 1ml 0.1M HCl/Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (0.1% w/v).

(g) The vials were placed in a chromatographic oven overnight for drying at 75°C. Next day, 5ml ACS II was added to the vials, mixed for 30s and counted on a  $\beta$ -counter after leaving them at room temperature for >1hr.

### 3.5 Calculations:

The recommended unit of an enzyme activity is the 'Katal' which is defined as the amount of enzyme that converts one mole of substrate per second and specific activity as 'Katal' per killogram, but it has commonly been used as pmole per hour and specific activity pmole per hour per milligram by other workers (Saavedra et al., 1974b; Pendleton et al., 1977; Luine and McEwen, 1983). This commonly used unit had been used throughout in this report.

Activity was expressed as pmol of phenylethanolamine-N-[methyl-<sup>3</sup>H] formed per hour (pmol/h) and Specific Activity as pmole per hour per milligram (pmol/h/mg). This was evaluated by using a pre-programmed computer [Apple II]. Since the counting efficiency was constant in the present assay, the requirement for the determination of absolute radioactivity (DPM) was unnecessary. however, two measurements were required in order to evaluate the activity;

(a) the total radioactivity (CPM) corresponding to the added hot SAM per tube. Since 2.92  $\mu$ Ci was added to the assay tubes, this amount of hot SAM was added to scinillation vials containing 5ml ACS II and counted on a Packard  $\beta$ -counter. This gave 2051911 CPM (total radioactivity).

(b) the total amount of SAM. Since both cold and hot SAMS were added to each tube, the total amount of SAM = cold + hot; 185pmol + 193 pmol = 378 pmoles [total amount of SAM]. From these two measurements specific activity of PNMT for pheylethanolamine can be calculated

provided always that the fraction of the radioactivity (corrected CPM) appearing with the product, pheylethanolamine-N-methyl-[<sup>3</sup>H] and protein content of the sample are known.

The procedure for calculating specific activity is outline below:

(a) subtract the 'tissue blank' values (background CPM) from the fraction of the radioacdtivity appearing with product (unknown samples CPM) [unknown samples CPM-background CPM = corrected CPM];

(b) multiply the corrected CPM with total amount of SAM [corrected CPM x 378 pmoles] and then divide it by the total radioactivity [2051911];  
 PNMT activity =  $\frac{\text{corrected CPM} \times 378}{2051911}$  pmol/hr since the tissue was incubated per hour at 37°C;

(c) finally the results can easily be converted to specific activity by dividing it with protein content (mg) of the sample.

Therefore,

$$\text{Specific activity} = \frac{\text{corrected CPM} \times 378}{2051911 \times \text{Protein(mg)}} \text{ pmol/hr/mg}$$

### 3.6 Precision, Sensitivity and Specificity

Precision was determined by the inter and intra-assay coefficients of variation and these were 13% and 7.8% respectively. The sensitivity (2 x S.D. of 'tissue blank' value) of this assay was 1pmol/hr/mg protein. Since in the assay described in this section only negligible [<sup>3</sup>H]counts per minute were obtained when using tryptamine or pheylethylamine as substrate, in contrast to β-hydorxypheylethylamine, the assay is highly specific for PNMT. Furthermore, it mimics the in vivo situation in which noradrenaline containing a hydroxyl group at β-position is N-methylated to adrenaline by the brain PNMT.

### Chapter Three

#### ELICITATION OF THE LH SURGE IN IMMATURE RATS IN RESPONSE TO OESTROGEN

##### Introduction:

It is generally accepted that hypothalamo-hypophysial-gonadal negative feedback mechanisms in both male and female rats are functional during the neonatal stage of development (Goldman et al., 1971; Eldridge et al., 1974). However, mechanisms responsible for a gonadotrophin positive feedback response to an oestrogen stimulus are synchronized on the day preceding the first natural onset of ovulation in young females (Meijs-Roelofs et al., 1975), whereas male rats are incapable of such a response at any age (Döhler & Wuttke, 1974).

In short-term (acutely ovariectomized rat model) ovariectomized adult rats (Mackinnon et al., 1985) and long standing ovariectomized adult rats (Calgaris et al., 1971; Burnet & Mackinnon, 1975), a massive output of gonadotrophin (LH) similar to that seen in intact cyclic proestrous rats can be elicited by treatment with 17  $\beta$  -oestradiol-3-benzoate (OE<sub>2</sub>, Sigma Chemicals Co. Ltd., U.K.). Furthermore, immature rats of 27-days of age and under 60g body weight when treated with pregnant mare serum gonadotrophins (PMSG) showed an LH surge 52-54h after an initial injection (s.c) of PMSG, but they do not ovulate (ter Haar & Wilson, 1978). Similarly, in a previous ontological study of the OE<sub>2</sub>-stimulated gonadotrophin surge, it was found that the occurrence of this neuro-humoral event was critically related to the age and sex of the animal. It could be elicited in 21-day-old females, but not in 16-day-old females or in males of any age (Puig-Duran & Mackinnon, 1978b); these 21-day-old animals, however, do not ovulate. The preliminary experiments presented in this chapter were undertaken to confirm earlier findings in our present colony of rats since this was a model that I wished to use for further studies (see Chapters 4 and 5).

## Materials and Methods

### Animals:

Wistar rats of both sexes aged 16, 18, 20, 21 and 22 days were used in this study. They were taken from litters which had been culled, within 24h of birth, to a total of 8 (male and female) pups. Each litter was kept with their mother in a single cage until they were weaned at 21-days of age. Lighting and temperature in the animal house were automatically regulated (lights on 0600h: lights off 2000h; temperature  $22 \pm 2^\circ\text{C}$ ), and food (Diet FFG; Dixon & Sons, Ware) and water were freely accessible to the animals.

### Experimental procedures:

1. Groups of 16, 20, 21 and 22-day-old male and female rats were injected (s.c) through the scruff of the neck with  $1\mu\text{g}$   $\text{OE}_2$  in 0.1ml arachis oil:ethyl oleate (4:1, v/v) at noon on experimental day 1 (as described by Puig-Duran & Mackinnon, 1978a) and decapitated at either 1000h or 1800h on day 3. Previous studies have shown that these are the times when minimum (1000h) and maximum (1800h) concentrations of serum LH are found (Puig-Duran & Mackinnon, 1978a,b).
2. 22-day-old male and female rats were injected (s.c.) with oil or  $\text{OE}_2$  at noon on experimental day 1 and decapitated at either 1500h, 1600h, 1700h, 1800h or 1900h on day 3.

### Collection of blood samples:

Blood samples were taken by draining the trunk after rapid decapitation. The blood was allowed to clot in a PTF 1289 plastic tube for 4-5hr in the cold ( $4^\circ\text{C}$ ) and then centrifuged at  $4^\circ\text{C}$  for 15min. The serum was then collected into LP2 tubes, frozen, and stored at  $-20^\circ\text{C}$  until the time of assay.

### Measurements of concentrations of LH in serum samples:

Serum LH concentrations were measured by an ovine-ovine radioimmuno assay



(RIA) as described in Section 'A' of Chapter 2.

### Results:

Serum LH concentrations (NIH-LH-S13,  $\mu\text{g/l}$  in  $\text{OE}_2$ -treated 16, 18, 20, 21 and 22-day-old male and female rats:

Immunoreactive serum LH concentrations in infant male rats (16-22 days of age) injected (s.c.) with  $\text{OE}_2$  on experimental day 1 and decapitated on day 3 at either 1000h or 1800h were baseline (Table II). Similarly, 16, 18-day-old  $\text{OE}_2$ -treated (day 1) female rats showed basal levels at either 1000h or 1800h on day 3, with the exception of two 18-day-old animals which showed considerably higher concentrations of LH at 1800h (Table III). With respect to the groups of 20, 21 and 22-day-old female rats, LH concentrations were at basal level at 1000h, while all the animals showed much higher LH levels at 1800h; with the exception of two 20-day-old rats which showed baseline values (Table III). The concentrations of immunoreactive LH in the serum of 22-day-old female rats injected with  $\text{OE}_2$  on day 1 were much higher at 1800h than at 1700h or 1900h (Table V). There were no differences in serum LH concentrations at either 1700h, 1800h or 1900h in similarly treated 22-day-old male rats, (Table V) and oil injected (s.c.) 22-day-old female rats, (Table IV).

### Discussion:

It could be argued that jugular venepuncture under ether anaesthesia is preferable to collection of trunk blood at decapitation, since it would have enabled both blood samples (1000h and 1800h) to be taken from the same animal. However, previous studies have shown that jugular venepuncture which involves both handling and anesthesia prior to bleeding causes a complete disappearance of episodic bursts of high LH levels observed in immature rats of both sexes (between 4 days and the onset of puberty) (Mackinnon et al., 1976). Furthermore, it is generally accepted that rapid decapitation minimises the effects of stress (e.g.

16-day-old			18-day-old			20-day-old			21-day-old			22-day-old							
1000h	1800h		1000h	1800h		1000h	1800h		1000h	1800h		1000h	1800h						
NO.	LH	NO.	LH	NO.	LH	NO.	LH	NO.	LH	NO.	LH	NO.	LH	NO.					
1	0.39	1	0.46	1	0.62	1	5.56	1	1.13	1	0.49	1	0.38	1	0.84	1	0.71	1	0.8
2	0.52	2	0.90	2	ND	2	0.49	2	1.05	2	0.30	2	0.72	2	1.06	2	0.90	2	0.3
3	0.35	3	ND	3	ND	3	5.08	3	0.39	3	0.26	3	0.27	3	1.48	3	0.74	3	ND
4	0.40	4	0.59	4	0.44	4	3.57	4	0.49	4	0.42	4	0.49	4	1.35	4	9.96	4	1.5
5	0.28	5	0.29	5	0.30	5	2.98	5	0.29	5	0.55	5	0.42	5	0.62	5	0.64	5	0.6
		6	1.17	6	0.36	6	1.68			6	0.59			6	0.55			6	0.3
		7	0.63															7	3.9

Concentration of immunoreactive LH ( $\mu\text{g/l}$ ) in the serum of immature female rats injected (s.c.) with  $1\mu\text{g}$  oestradiol benzoate ( $\text{OE}_2$ ) at 1200h on day 1 and decapitated at either 1000h or 1800h on day 3

[illegible]

Table IV

Concentration of immunoreactive LH ( $\mu\text{g/l}$ ) in the serum of 22-day-old female rats injected (s.c.) with oil [arachis oil: ethyl oleate, 4 : 1 (v/v)] at 1200h on day 1 and decapitated at 1500h, 1600h, 1700h, 1800h or 1900h on day 3

1500 h		1600 h		1700 h		1800 h		1900 h	
No.	LH	No.	LH	No.	LH	No.	LH	No.	LH
1	1.65	1	0.62	1	0.28	1	0.37	1	0.36
2	0.34	2	0.76	2	0.84	2	0.25	2	0.39
		3	0.26	3	1.69	3	0.25	3	1.78
		4	0.29	4	0.32	4	0.46		

Profiles of the concentration of immunoreactive LH ( $\mu\text{g/l}$ ) in the serum of immature rats injected (s.c.) with  $1\mu\text{g}$  oestradiol benzoate ( $\text{OE}_2$ ) at 1200h on day 1 and decapitated at either 1700, 1800 or 1900h on day 3

22-day-old female ( $\text{Q}$ ) rats

1700 h		1800 h		1900 h	
No.	LH	No.	LH	No.	LH
1	0.45	1	> 32.00	1	2.44
2	1.99	2	> 32.00	2	10.96
3	0.25	3	> 32.00	3	> 32.00
4	1.29	4	> 32.00	4	22.30
5	0.99	5	> 32.00	5	10.58
6	1.12	6	> 32.00		
7	0.63	7	6.40		
8	12.55	8	> 32.00		
9	1.10	9	> 32.00		
		10	> 32.00		

22-day-old male ( $\text{O}$ ) rats

1700 h		1800 h		1900 h	
No.	LH	No.	LH	No.	LH
1	1.43	1	0.80	1	0.37
2	0.46	2	0.36	2	0.60
3	1.91	3	ND	3	ND
4	0.28	4	1.57	4	1.01
5	0.33	5	0.68	5	0.27
6	ND	6	0.35		
7	0.32	7	3.90		

Döhler et al., 1976).

Measurements of LH concentrations in oil-treated female rats (see Table IV) did not show an increased LH surge and thus served as a 'control group'. Serum LH concentrations in 2 out of 6 of the OE<sub>2</sub>-stimulated 18-day-old female rats at 1800h on day 3 were considerably higher than basal levels (see Table III). However, these LH levels cannot be considered as an LH surge since they were much lower than that of endogenous oestrogen-stimulated proestrous rats (Mackinnon et al., 1983). Of the experimental OE<sub>2</sub>-treated 20-day-old female rats, 3 out of 5 showed a surge output of LH at 1800h on day 3, while 2 showed only basal levels (see Table III). A clue to the possible explanation for this observation that a proportion of 18-day, and 20-day-old experimental animals respectively showed a considerable increase in the concentrations of LH as compared to their litter mates, comes from a previous study (ter Haar & Wilson, 1978). In that study both lighter (<60g) and heavier (>60g) weight 27-day-old animals when treated with PMSG on experimental day 1 showed a surge of LH with a peak at 1800h on day 3, as measured by the heterogenous radioimmuno assay (ovine-ovine RIA, described in materials and methods). However, similarly treated, heavy weight animals showed a surge of LH on day 3, as measured by the homologous RIA (rat anti-serum, purified rat LH for iodination and rat LH standards-system) and furthermore, these animals ovulated on experimental day 4 (Wilson et al., 1985). It has been suggested that a surge of LH which was shown by lighter animals might be a pleiomorphic form which is inactive and, consequently did not cause ovulation. Whereas a change in the structure of LH, (active form), in association with the weight (>60g) of animals might be responsible for causing the ovulation. Furthermore, using this PMSG-treated prepubertal rat model it was suggested that

the influence of body weight might play a critical role in the onset of puberty (Wilson et al., 1983). If this were the case then it is likely that a proportion of both 18, and 20-day-old females which showed a considerably high level of LH might also have had higher body weight as compared to their litter mates.

Possibly, it can be said that body weight of these animals might be worth taking into consideration during the course of such studies and this was followed in the remaining studies presented in this thesis.

With respect to 16-day-old female rats (Table III) the failure of a gonadotrophin response to an oestrogen stimulus as compared to 22-day-old females could have been due to the presence of much higher levels of  $\alpha$ -foeto-protein which has a high affinity for oestrogen (Raynaud et al., 1971). This protein, which is produced in foetal liver, declines steadily from birth to 21-days of age (Nunez et al., 1976). However, in rats treated with diethylstilboestrol, a synthetic oestrogen which does not bind avidly to  $\alpha$ -foeto-protein, the gonadotrophin positive feedback response was also prevented (Puig-Duran & Mackinnon, 1978b). An explanation as to why these animals did not show a gonadotrophin positive feedback response still remains to be ascertained.

The profiles of the concentrations of LH in the 22-day-old males and females showed that peak values occurred at 1800h on day 3, while none of the similarly treated male rats showed a gonadotrophin response to an oestrogen stimulus (Table V). Furthermore, in the present study, none of the experimental male rats (16-22 days of age) responded to oestrogen with a release of LH at 1800h on day 3 (Table II). This is in agreement with previous studies in which the presence of oestrogen or testosterone during a developmental critical period (i.e. within 5 days post-partum) was shown to masculinize the brain and therefore

prevent the LH surge response to an oestrogen stimulus at any age (Barracclough, 1961; Gorski, 1963).

In conclusion, serum LH levels of OE<sub>2</sub>-treated 16 and 21 or 22-day-old females and males of 16-22 day of age measured at both 1000h and 1800h on day 3 are compatible with those shown in a previous study (Puig-Duran and Mackinnon, 1978a, b).



## Chapter Four

### CHANGES IN ADRENALINE CONCENTRATIONS IN THE MEDIOBASAL HYPOTHALAMUS OF IMMATURE RATS INDUCED WITH OESTROGEN.

#### Introduction:

Recently, a series of studies using an inhibitor, (SKF64139) of the centrally active adrenaline synthesizing enzyme (PNMT) has indicated that adrenaline might play a critical role in the output of the pre-ovulatory surge of LH (Crowley & Cass-Terry, 1981; Crowley et al., 1982; Coen et al., 1983). Investigations of adrenergic activity in the MPOA and MBH of regularly cyclic proestrous rats have shown that activity increases during the neural 'critical period'. Moreover, it is oestrogen-dependent and can be blocked by pentobarbital (Mackinnon et al., 1983; 1985). These investigations supported the suggestion that steroid-stimulated adrenergic activity in the MBH may be concerned with the preovulatory release of gonadotrophins.

It has been previously observed, and also confirmed by the present studies (see Chapter 3), that 21-, or 22-day-old female rats if injected (s.c.) with oestradiol benzoate (OE<sub>2</sub>) at noon on experimental day (day 1) show an LH surge 54h later, at 1800h on day 3. Moreover, this surge in output of LH is similar in timing and magnitude to that of the cyclic proestrous and chronically ovariectomized oestrogen-stimulated adult rats. Similar treatment of either 16-, 18-, 20-, 21-, and 22-day-old males or 16-day-old females has not shown such a response. The experiments presented in this chapter were undertaken to ascertain whether central adrenaline is indispensable for the production of LH surge on OE<sub>2</sub>-treated immature rats. Adrenaline concentrations were measured in the MPOA and MBH of infant rats at pertinent times after an initial injection of OE<sub>2</sub>.

## Materials and Methods

### Animals:

Wistar rats of both sexes aged 22 days (mean body weight (b.w.)  $46 \pm 2$  (S.E.M.) g) and 16-day-old females ( $25 \pm 1$ g (b.w.)) were used in this study. Care and handling of the animals have been described in Chapter 3.

### Experimental procedures:

1. 22-day-old males and females were injected (s.c.) with  $1\mu\text{g}$   $\text{OE}_2$  in 0.1ml arachis oil:ethyl eoleate (4:1, v/v) at noon on experimental day (day 1) (as described by Puig-Duran & Mackinnon, 1978a) and decapitated at hourly intervals between 1200h and 1800h of day 3.
2. 22- and 16-day-old female rats were injected with  $1\mu\text{g}$   $\text{OE}_2$  on experimental day 1 and decapitated at either 1500, 1600 or 1700h of day 2 and day 3 respectively.

The MPOA and MBH samples were removed as described previously (see Section 'B' of General Materials & Methods) and homogenized either in 25 $\mu\text{l}$  0.1M HCl solution containing 1mM diethylenetriaminepenta acetic acid and 0.1% sodium metabisulphite (see Section 'C' of General Materials & Methods) for subsequent measurements of adrenaline.

### Catecholamine concentrations in the MPOA and MBH

A radioenzymatic assay was used for measurements of DA, NA and A in the MPOA and MBH. This assay with small modifications, was that described by Saller & Zigmond (1978) (see Section 'C' of General Materials & Methods for details).

### Glassware:

Glass tubes were used for the radioenzymatic assay of catecholamines. These tubes were silanized by exposing them to the fumes of dimethyldichloro-

silane in a fume-tight vessel for a period of at least 24hrs. Before use they were rinsed with distilled water and dried in an oven.

#### Expression and statistical analysis of results:


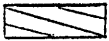
The results mentioned in this chapter are expressed in terms of total tissue protein, the form which is most commonly encountered in the literature. However, some authors have expressed their results in terms of wet weight (Glowinski & Iversen, 1966). It was not feasible to exploit this technique since the frozen samples of MPOA and MBH were too small to handle accurately without loss of weight. The protein in tissue samples was measured by the method of Lowry et al., (1951) (see Appendix for details).

Statistical significance of differences between group means was assessed with a Newman-Keuls test, if warranted following analysis of variance (Winer, 1971). A value for  $P \leq 0.05$  was regarded as significant.

#### Results:

##### Concentrations of catecholamines (ng/mg Protein in the MBH and MPOA of oestrogen-treated infant rats:

Mean concentrations ( $\pm$ S.E.M.) of adrenaline (ng/mg Protein) in the MBH of both 22-day-old male and female rats treated with  $OE_2$  on experiemntal day 1 were at baseline levels between 1200h - 1800h on day 3 (Fig.15). The exception, however, was that of adrenaline concentrations in the MBH of females at 1600h which were significantly higher at 1600h than at either 1500h ( $P < 0.01$ ) or 1700h ( $P < 0.01$ ) both on days 2 and 3 (Fig.17). The concentrations of adrenaline in the MPOA of these rats of either sex also showed basal levels at 1500h, 1600h and 1700h on day 3 (Fig.16). Likewise, similarly treated 16-day-old female rats showed basal levels of adrenaline in the MBH at 1500h, 1600h and 1700h on day 3 (Fig.17).

Fig.15. Mean adrenaline (A) concentration ( $\pm$ S.E.M.) in the mediobasal hypothalamus of 22-day-old male (  ) or female (  ) rats injected (s.c.) with 1 $\mu$ g oestradiol benzoate (OE<sub>2</sub>) at 1200h on experimental day 1. Groups of animals were decapitated at one-hourly intervals between 1200h and 1800h on day 3. The number of animals used is shown in brackets.

\*\* 1600h : 1500h ( $P < 0.01$ )

1600h : 1700h ( $P < 0.01$ )

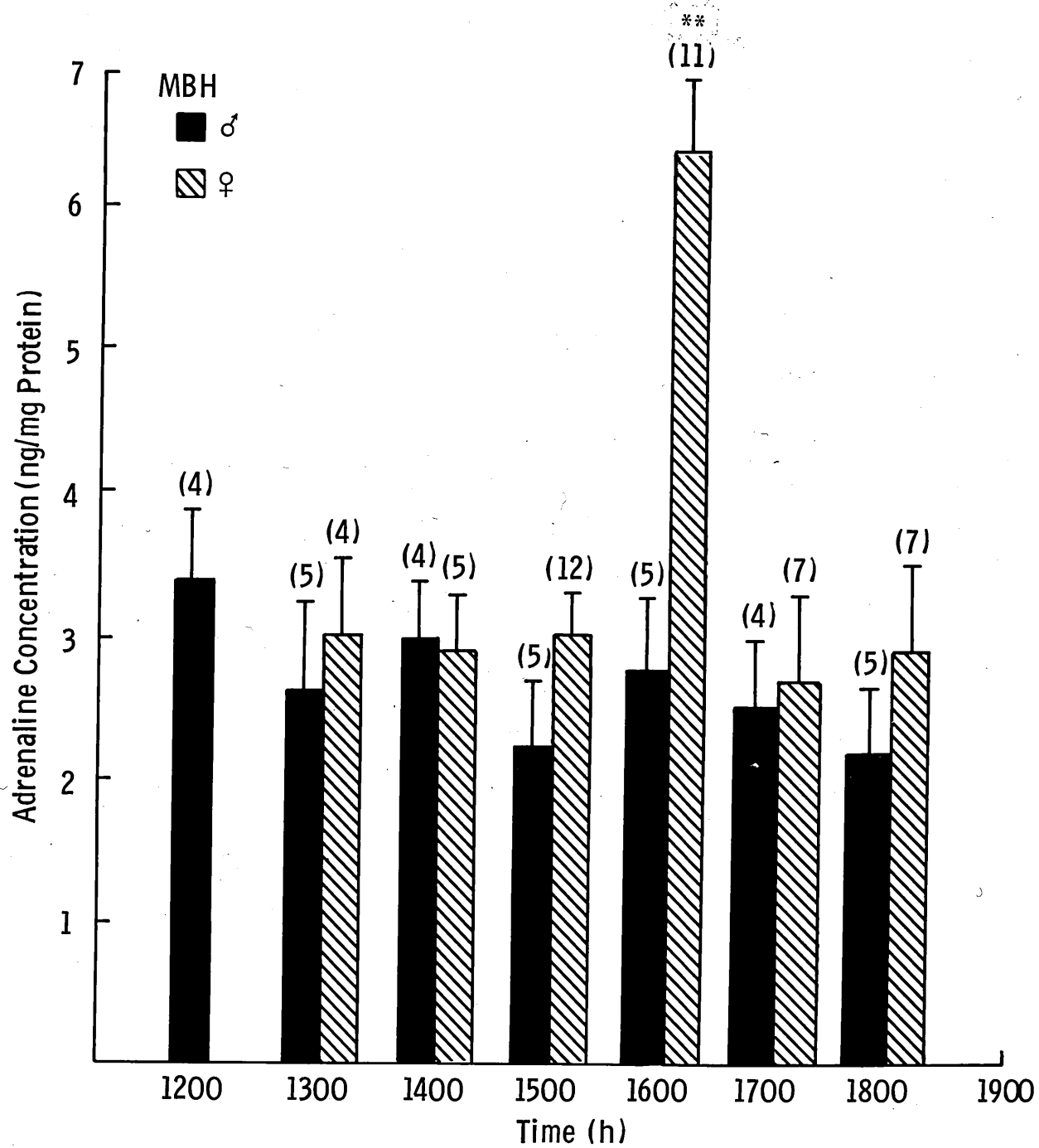

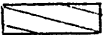


TABLE VI

Mean adrenaline concentrations ng/mg ( $\pm$  S.E.M.) in the mediobasal hypothalamus of 22-day-old female rats injected with oil [arachis oil: ethyl oleate, 4:1 (v/v)] at 1200 h on experimental day 1. Groups of animals were decapitated at 1500h, 1600h or 1700h on day 3. The number of animals used is shown in brackets

1500 h	1600 h	1700 h
$3.66 \pm 0.21$	$3.00 \pm 0.39$	$1.91 \pm 0.26$
(3)	(4)	(4)

Fig.16. Mean adrenaline (A) concentrations ( $\pm$ S.E.M.) in the medial preoptic area of 22-day-old male (  ) or female (  ) rats injected (s.c.) with 1 $\mu$ g oestradiol benzoate (OE<sub>2</sub>) at 1200h on experimental day 1. Groups of animals were decapitated by 1500h, 1600h or 1700h on day 3. The number of animals used is shown in brackets.

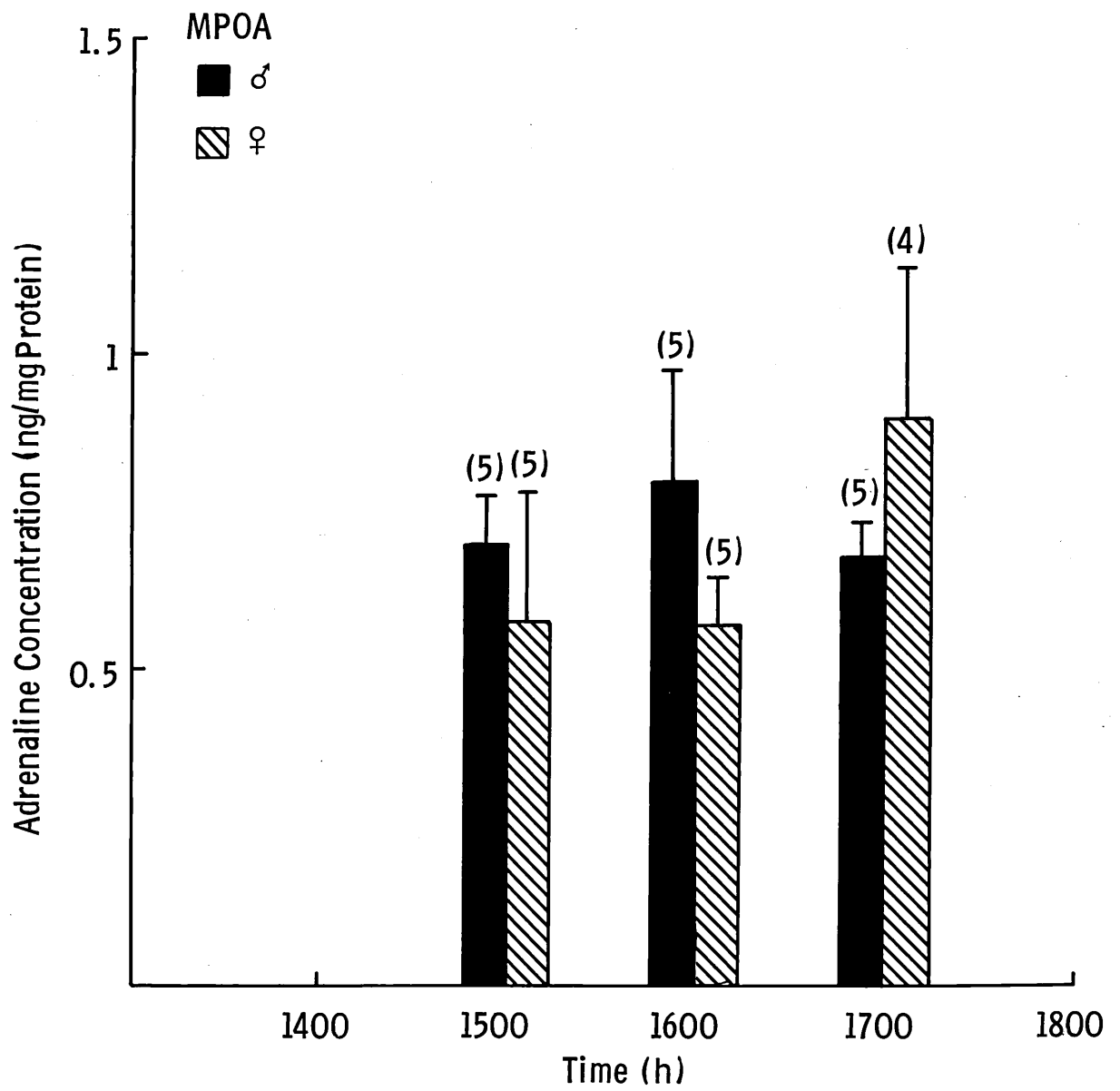






Fig.17. Mean dopamine (DA), noradrenaline (NA) and adrenaline (A) concentration ( $\pm$ S.E.M.) in the mediobasal hypothalamus of 16-day-old (  ) or 22-day-old (  ) female rats injected (s.c.) with 1 $\mu$ g oestradiol benzoate (OE<sub>2</sub>) at 1200h on day 1. Groups of animals were decapitated at 1500h, 1600h or 1700h on either day 2 or day 3. The number of animals used is shown in brackets.

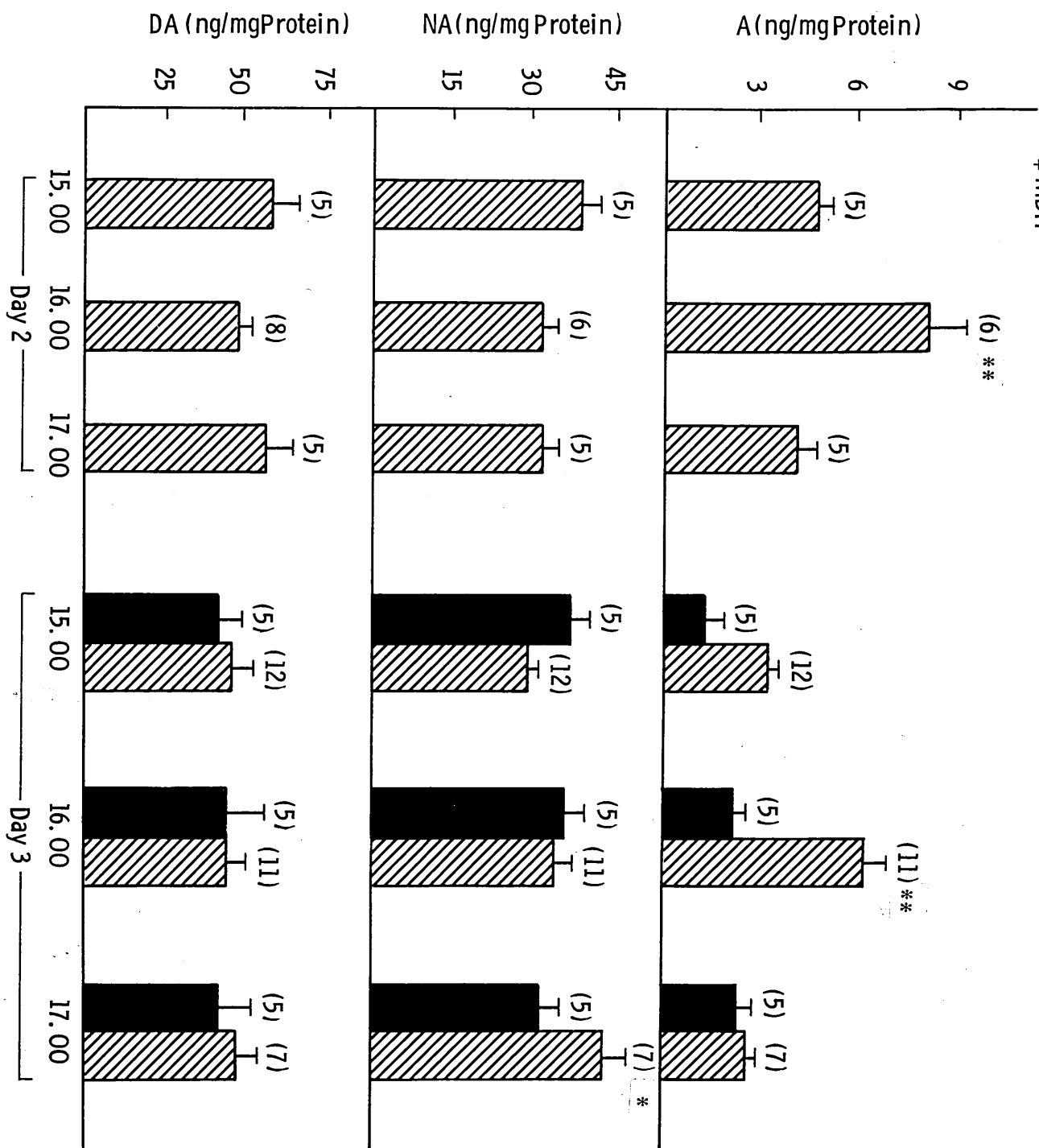
\* 1700h : 1500h (P<0.05)

1700h : 1600h (P<0.05)

\*\* 1600h : 1500h (P<0.01)

1600h : 1700h (P<0.01)

♀ MBH



There were no significant differences in the concentrations of either DA or NA in the MBH of OE<sub>2</sub>-treated 16, and 22-day-old female rats decapitated at 1500h, 1600h or 1700h on either day 2 or day 3; with the exception of NA concentrations in the MBH of 22-day-old rats at 1700h on day 3 which were significantly higher than at 1500h ( $P < 0.05$ ) or 1600h ( $P < 0.05$ ) (Fig.17). There were no significant differences in the concentrations of A in the MBH of oil-treated 22-day-old female rats decapitated at 1500h, 1600h or 1700h on day 3 (Table VI).

### Discussion:

The turnover of a metabolite in a cell refers to that amount which is transported or metabolized. It is generally accepted that measurements of turnover rate of a neurotransmitter is a sensitive index of its functional activity (Pycock & Taberner, 1981). In order to measure the turnover of catecholamines, two main types of method have previously been used, namely, Radioisotopic labelling and Enzyme inhibition. In the radioisotopic method a suitable labelled precursor is introduced into the brain either by infusing into the ventricle or through the bloodstream. The rate of change of the amount of this isotopically labelled substance present in the brain is subsequently measured. The use of this method came from a study in which labelled noradrenaline ( $0.22\mu\text{g}$ , [<sup>3</sup>H]-noradrenaline, Sp.activity 5 Ci/mmol) or dopamine ( $1.5\mu\text{g}$  [<sup>3</sup>H]-dopamine, Sp.activity 1.5 Ci/mmol) were injected in a volume of 20 - 30 $\mu\text{l}$  into the lateral ventricle of the rat brain by stereotaxis to avoid the blood-brain barrier, (Iversen & Glowinski, 1966). These research workers determined the half life values [ $t_{1/2} = (\log_{10}^2)/\text{slope}$ ] from the initial rate of decline of labelled noradrenaline, or noradrenaline synthesized from labelled dopamine in different parts of the brain. The turnover noradrenaline which is the product of the half life and the initial concentration of labelled noradrenaline in the tissue was then calculated. However, radioisotopic labelling is

open to criticism. The criticism is that not all of the catecholamine injected into the lateral ventricles is taken up by the appropriate nerve terminals. Such non-specific radioisotopic labelling might induce artifacts. Furthermore, the intraventricular addition of labelled catecholamine might saturate the endogenous amine pools; and in turn this might consequently alter the kinetics of the enzymes responsible for synthesizing or metabolizing these amines.

In the second method which pertains to enzyme inhibition, either the activity of a neurotransmitter metabolizing enzyme or that of its synthesizing enzyme is blocked; thereafter, the accumulation of neurotransmitter or the decline in its concentration are measured respectively. However, the measurements of adrenaline or its metabolite, metanephrine as an index of adrenergic activity after monoamine oxidase inhibition has not been feasible due to a feedback effect on the synthesizing enzyme (PNMT) (Fuller & Hunt, 1967). The turnover rate of adrenaline in discrete regions of the rat brain, as measured by the decline in concentration of adrenaline following inhibition of PNMT has been reported (Sauter et al., 1978; Fuxe et al., 1979). However, turnover measurements of adrenaline have not been made in the present studies since pharmacological suppression of catecholamine transmission is also open to criticism. One piece of evidence indicated that inhibitors of PNMT possess  $\alpha$ -adrenergic receptor antagonist activity (Toomey et al., 1981) and in addition, inhibit monoamine oxidase activity (an enzyme responsible for metabolising catecholamines) (Fuller et al., 1983). It is possible therefore that increased turnover of catecholamines shown by previous studies might be due to receptor antagonism and/or monoamine oxidase inhibitory activity of the pharmacological agents used. Furthermore, an increased turnover of NA, as measured by its

rate of decline following the administration (i.p.) of a drug ( $\alpha$ -Methyl-p-tyrosine,  $\alpha$ -MPT) which inhibits tyrosine hydroxylase, was consistently accompanied by an increase in baseline levels of NA (Rance et al., 1981; Hiemke et al., 1985). Similarly in the MBH of adult rats, an increased turnover of adrenaline (as measured by its rate of decline, 2h after following a drug, SKF64139 induced inhibition of PNMT) was invariably seen to accompany an increase in baseline concentrations (Sheaves et al., 1985a). This suggests that an increased turnover of a catecholamine found in previous studies was merely due to enhanced baseline values.

Using 30-day-old female rats injected (s.c.) with pregnant mare serum (PMS) on experiment day 1, it has been shown that ovulation can be elicited about 64h later, on day 4 (Strauss & Meyer, 1962). Furthermore, the animals were injected (i.p.) with barbiturate at different times during day 3, in order to determine a neural 'critical period'. It was found that the time of a neural 'critical period' in this PMS treated prepubertal rat model was similar to that of adult rats (Everett & Sawyer, 1950). Therefore, it is likely that a neural 'critical period' in 22-day-old female rats treated with OE<sub>2</sub> on day 1, occurs at the same time on day 3 as in adult proestrous rats of our colony ('critical period', 1430-1700h).

An increased adrenaline concentration during the 'critical period' in the MBH of OE<sub>2</sub>-treated (day 1) 22-day-old female rats (decapitated at 1600h on day 3 which precedes the LH surge), was absent in the MPOA of these animals (Fig.16). These findings are in agreement with a previously reported study in which it has been shown that an OE<sub>2</sub>-stimulated increase in adrenaline concentrations in the MBH of acutely ovariectomized adult rats, occurred during the 'critical period',

followed about 2h later by an LH surge. Infant male rats under similar treatment did not respond to an OE<sub>2</sub>-stimulus in releasing the output of LH and furthermore, did not show an increase in the concentration of adrenaline in the MBH at 1500h, 1600h or 1700h on day 3 (Fig.15). On the basis of these observations, it is tempting to suggest that an increased adrenaline concentration in the MBH of OE<sub>2</sub>-treated 22-day-old females on day 3 might be involved in the mechanism underlying the critical period which is presumably associated with the output of GnRH and hence the LH surge. Whether the increase of adrenaline concentration in the MBH represents an increased synthesis of the neurotransmitter or an accumulation of its concentration, due perhaps to a decrease in its release or decreased monoamine oxidase/COMT activity is not known. Moreover, the precise location of the increased concentrations of A in the MBH of infant rats is not known, although measurements of A in the arcuate nucleus and in the medial and lateral aspects of the median eminence of cyclic proestrous rats have shown that at least 80% is concentrated in the ME, mainly in its lateral aspects (Sheaves et al., 1984). According to Rance et al., (1981), increased noradrenergic activity in the MPOA might represent the neural mechanism underlying the neural 'critical period'. A further study by Rance & Barraclough (1981) concerned with the measurement of noradrenergic activity in the MPOA of pentobarbital blocked adult rats did not support this suggestion, since it was observed that the noradrenergic activity was not significantly affected by pentobarbital blockade. The present study of 22-day-old female rats OE<sub>2</sub>-treated on experiment day 1 and decapitated at either 1500h and 1600h or 1700h on day 3 did not show an increase in either A and NA or DA concentration in the MPOA. Furthermore, an increased noradrenergic activity in the MBH at proestrous (1200-1500h) concomitant with a fall in the hypothalamic content of GnRH and an increase in

plasma LH levels, have also been reported and interpreted as representing processes involved with the initiation of the LH surge (Rance et al., 1981). However, barbiturate injected in an ovulatory-blocking dose at the start of this period (1200h) failed to effect this noradrenergic activity (Rance & Barraclough, 1981). On the other hand, adrenaline turnover in the MBH has been shown to be diminished significantly between 14.30 and 16.30h (during the critical period) in proestrous rats which had been injected with pentobarbital (Sagatal; i.p., 36mg/kg) in an ovulatory-blocking dose at the start of the critical period (Mackinnon et al., 1983).

To ascertain whether increased adrenaline concentrations in the MBH are specifically associated with the output of LH surge on day 3, catecholamine concentrations were measured in the MBH of 22-day-old female rats injected with OE<sub>2</sub> on experimental day 1 and decapitated at either 1500h and 1600h or 1700h on day 2, which does not seem to be associated with the output of LH. It was found that mean concentrations of adrenaline were significantly higher at 1600h than at either 1500h ( $P < 0.01$ ) or 1700h ( $P < 0.01$ ) similar to day 3, while DA and NA concentrations were at baseline level (Fig.17). These observations might indicate that an increased adrenaline concentration in the MBH preceding the LH surge on day 3 might not be specifically associated with its output. In the previous measurements of serum prolactin concentrations in 21-day-old female rats using the same experimental regime, it has been shown that prolactin concentrations were significantly higher at 1800h than at 0900h both on days 2 and 3 (Puig-Duran & Mackinnon, 1978a). It is generally accepted that tubero-infundibular dopamine is one of the factors which inhibits the output of prolactin at the anterior pituitary level (reviewed by Leong et al., 1983). An important piece of evidence came from a study in which the concentrations of dopamine in the hypothalamo-hypophysial portal blood were measured during the oestrous cycle (Ben-Jonathan et al., 1977). It has

been found from this study that the pro-oestrous surge of prolactin is accompanied by a fall in dopamine level in the portal blood. A fall in dopamine in the portal blood probably reflects its release having been inhibited into the portal vessels. If this were the case then it should be possible to measure an accumulation of dopamine in the MBH. However, in the present study variations in the concentrations of dopamine in the MBH of 22-day-old female rats OE<sub>2</sub>-treated on day 1 were not found either on experimental day 2 or 3 (Fig.17). It is possible that the accumulation in the concentration of dopamine is not sufficient to be detectable.

There is growing evidence to suggest that thyrotrophic-releasing hormone (TRH) might be one of the factors responsible for the stimulation of the proestrous output of prolactin (reviewed by Leong et al., 1983). In support of this hypothesis an increase in the number of TRH receptors on the lactotrophs has been found on proestrus (Delean et al., 1977). Furthermore, measurements of TRH concentrations in the hypothalamo-hypophysial portal vessels during the oestrous cycle showed that TRH increased on the afternoon of proestrus (Franks et al., 1984). Based on these findings, it has been proposed that oestrogen influences the output of TRH into pituitary portal blood which interacts with lactotrophs in releasing prolactin (Frank et al., 1984). It is possible that the increased concentrations of adrenaline both on days 2 and 3 in the MBH of the OE<sub>2</sub>-treated infant model, may be temporally related with the output of prolactin, possibly by mediating the oestrogen response to TRH and thereby regulating its output into the portal vessels. However, it could be argued that adrenaline is a general modulator involved with the output of pituitary hormones and operating either at the level of the anterior pituitary or at the MBH. This suggestion is supported by two pieces of evidence:



(a) 22-day-old male rats injected with OE<sub>2</sub> at noon on experimental day 1 did not show an increased output of serum prolactin 30h later (i.e. 1800h on day 2) or both prolactin and LH 54h later at 1800h on day 3 (Puig-Duran & Mackinnon, 1978a), a finding that was confirmed with respect to the output of LH in the present study. Failure to respond to an OE<sub>2</sub>-stimulus in releasing the prolactin on day 2 or prolactin accompanied by LH surge on day 3 was associated with an absence of an increased adrenaline concentration in the MBH (Fig.15).

(b) With respect to the 16-day-old female rats, failure to respond to an oestrogen stimulus was again associated with an absence of an increased concentration of adrenaline in the MBH at 1500h, 1600h or 1700h on day 3 (Fig.17). However, the same argument pertaining to the presence of  $\alpha$ -foeto-protein can be implied as described previously (see Chapter 3).

Since there is considerable current interest in the possible roles of DA and NA in the control of hormonal output from the anterior pituitary (Weiner & Ganong, 1978; Barraclough et al., 1984) it was notable that concentrations of DA and NA in the MBH remained at baseline levels during the time of sampling (1500 - 1700h on days 2 and 3), with the exception of NA concentrations at 1700h which were significantly greater than those at 1500h or 1600h ( $P < 0.05$ , Fig.17). This finding may relate to the increase in NA turnover which Rance et al., (1981) consider it to be related to the proestrous surge.

In conclusion, an oestrogen-stimulated increase in adrenaline concentration in the MBH occurs not only in the proestrous adult and OE<sub>2</sub>-treated ovariectomized rat, but also in the OE<sub>2</sub>-treated immature female rat. This event at least in the proestrous adult and the OE<sub>2</sub>-treated ovariectomized rat is associated with a higher turnover of adrenaline and presumably increased adrenergic activity. It is also worth noting that in the proestrous rat

the timing of the short-lived adrenergic activity occurs during the neural critical period. This increase in adrenaline concentration which can be elicited at 21-days of age but not before, is also sexually differentiated. Since adrenaline activity in the MBH increases consistently 2h before an outpouring of anterior pituitary hormones, there would seem to be a strong temporal relationship between the two events. Whether or not there is a direct relationship remains to be investigated.

## Chapter Five

### INVOLVEMENT OF OESTROGEN AND CORTICOSTEROID IN INDUCING CHANGES IN PHENYLETHANOLAMINE-N-METHYL TRANSFERASE ACTIVITY IN THE MEDIOBASAL HYPOTHALAMUS OF IMMATURE RATS.

#### Introduction:

Using the oestrogen-stimulated infant rat model, it was found that a marked but short-lived increase in adrenaline concentration occurred in the MBH of 22-day-old female rat both on days 2 and 3, whereas 16-day-old females or 22-day-old males did not show such a response (see Chapter 4). This increase in adrenaline concentration is temporally related to an output of pituitary hormones 2h later. But whether this increase is due to an increase in its synthesis, a decrease in its release, or a decrease in monoamine oxidase and/or catechol-o-methyl transferase, is not known. However, the regional distribution of phenylethanolamine-N-methyl transferase (PNMT) in the rat brain as measured by immunohistofluorescence (Hökfelt et al., 1974, Lew et al., 1977) or PNMT activity (Saavedra et al., 1974b) is consistent with a parallel distribution of adrenaline concentrations (Koslow & Schlumpf 1974). Furthermore, the interpretation of turnover of adrenaline is open to criticism as described in Chapter 4. One way to alleviate this problem of interpretation is to measure PNMT activity.

In the light of the above observations the ontological and sexually-related changes in activity of PNMT were measured. Data from several reports have indicated that the activity of choline acetylase (the enzyme which synthesizes acetylcholine) in the rat brain can be affected by changing plasma concentrations of gonadal hormones (Luine et al., 1975; Muth et al., 1980; Luine & McEwen, 1983). It is possible that adrenergic mechanisms may participate in the mediation of oestrogen-dependant

gonadotrophin output by affecting PNMT activity.

It is well documented that adrenocorticotrophin (ACTH) interacts with specific receptors in the adrenal cortex to stimulate an output of glucocorticoids. Glucocorticoids diffuse through the adrenal medulla before entering the blood stream and thereby modulate medullary PNMT activity (Ciaranello et al., 1978; reviewed by Ungar & Phillips, 1983; and Carmichael & Winkler, 1985). It is possible therefore that glucocorticoids may play some role in changing the activity of central PNMT in the oestrogen-treated infant rat.

The experiments described in this chapter were also undertaken to explore these two possibilities that oestrogen and corticosteroid might influence the PNMT activity. PNMT activity was measured in the MBH of intact or adrenalectomized infant rats after treating them with oil, oestrogen or dexamethasone.

## Materials and Methods

### Animals:

22-day-old Wistar rats of both sexes with a mean body weight (b.w) of  $46 \pm 2$  (S.E.M.)g and 16-day-old females ( $25 \pm 1$ g, b.w.) were used in this study. The care and handling of the animals have been described in Chapter 3.

### Experimental Procedures:

1. 16-day-old females and 22-day-old males and females were injected (s.c.) with  $1 \mu\text{g}$  OE<sub>2</sub> in 0.1ml oil [arachis oil: ethyl oleate, 4:1 (v/v)] or oil vehicle only on experimental day 1, and decapitated between 1400 - 1800h on day 3.
  2. Groups of 22-day-old females were treated with oestrogen as described above and decapitated at 4-hourly intervals starting at 1200h on day 2 and ending at 2000h on day 3.
  3. Further groups of 22-day-old female rats were adrenalectomized [using a dorso-lateral abdominal approach under ether anaesthesia] on day 1 and thereafter allowed free access to either drinking water containing 1% glucose, 0.9% ethanol and 0.40mM dexamethasone or drinking water containing 0.9% ethanol and 1% glucose. This drinking fluid was made up of 16ml of dexamethasone to 0.9ml of absolute alcohol. This was then gradually added to 99.1ml of drinking water containing 1g of glucose, while being stirred continuously (personal communication, Dr. Julia Buckingham). These animals were injected (s.c) with  $1 \mu\text{g}$  OE<sub>2</sub> on day 2 and decapitated at either 1500h, 1600h or 1700h on day 4.
- Brains were removed and prepared for PNMT measurements as described

previously (Section 'D' of General Materials and Methods).

All glassware in this assay was silanized (see Chapter 4).

#### Statistical analyses:

Statistical treatment of the data was by analysis of variance for unequal groups followed by the Newman-Keuls procedure.  $P < 0.05$  was considered to be significant (Winer, 1971).

#### Results:

##### Mean specific activities of PNMT (Pmol/h/mg) in the MBH and MPOA of the oestrogen-treated infant rat:

Mean specific activity of PNMT (Pmol/h/mg Protein) in the MBH of oestrogen-treated 22-day-old male and female rats increased significantly ( $P < 0.01$ ) on day 3 from 1400 to 1600h after which they decreased from 1700 to 1800h (Fig.18). However, mean specific activities in the MPOA of these female rats were at baseline levels between 1500 - 1800h (Fig.19).

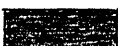
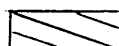
Measurements of mean specific activities of PNMT in the MBH of oil-treated 22-day-old male rats at 1500, 1600 and 1700h on day 3 showed a pattern similar to that of oestrogen-treated 22-day-old males and furthermore, there were no significant differences between the mean specific activities of PNMT in the oil, and oestrogen treated rats at any of these times (Fig. 20).

In contrast to oestrogen-treated 22-day-old females, there were no significant differences in the mean specific activities of PNMT in the MBH of oil-treated 22-day-old females or oestrogen-treated 16-day-old females at any time at which measurements were made (Tab.V).

Mean specific activities of PNMT in the MBH of oestrogen-treated 22-day-old female rats measured at 4-hourly intervals over days 2 and 3 showed a clear circadian rhythm. Peak levels of activity were reached at around 1600h and lowest levels at around 2400h (Fig.21).

Mean specific activities of PNMT (Pmol/h/mg) in the MBH of adrenalectomized dexamethasone-oestrogen-treated infant rats:

Mean specific activities of PNMT (Pmol/h/mg Protein) in the MBH of groups of adrenalectomized female rats treated with oestrogen and dexamethasone and decapitated at 1500h, 1600h and 1700h on day 4 were similar to those of oestrogen-treated intact 22-day-old females. However, similarly adrenalectomized oestrogen-treated 22-day-old female rats showed baseline levels of mean PNMT activities in the MBH at these times in the absence of dexamethasone in the drinking water (Fig.22).

Fig.18. Mean specific activity ( $\pm$ S.E.M.) of phenylethanolamine-N-methyl transferase (PNMT) in the mediobasal hypothalamus of 22-day-old male (  ) and female (  ) rats injected with 1 $\mu$ g oestradiol benzoate (OE<sub>2</sub>) at 1200h on experimental day 1. Groups of animals were decapitated at hourly interval between 1400 - 1800h on day 3. The number of animals used is shown in brackets.

1600h : 1400h ( $P < 0.01$ )

1600h : 1800h ( $P < 0.01$ )



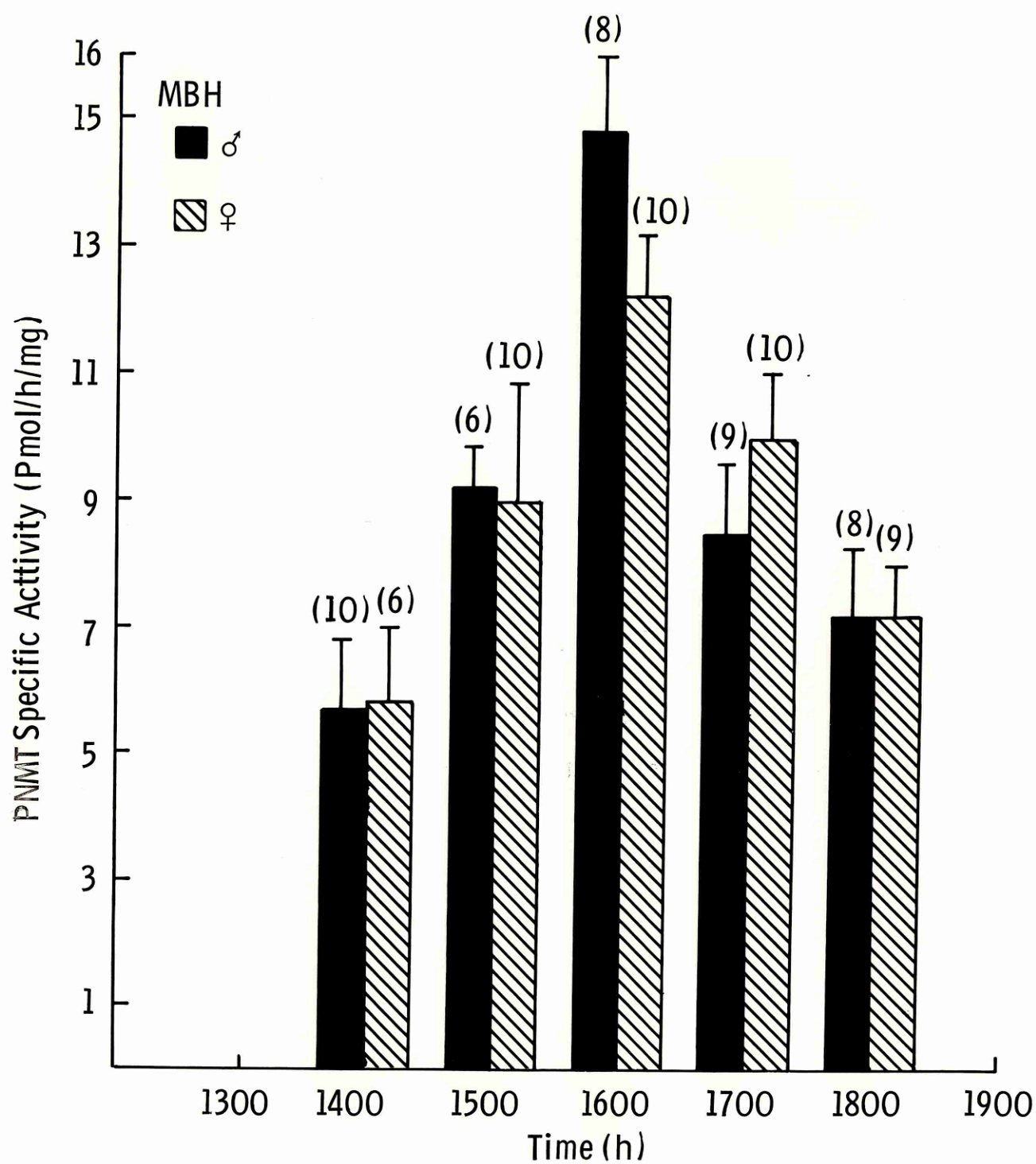


Fig.19. Mean specific activity ( $\pm$ S.E.M.) of phenylethanolamine-N-methyl transferase (PNMT) in the medial preoptic area of 22-day-old female rats injected with 1 $\mu$ g oestradiol benzoate at 1200h on experimental day 1. Groups of animals were decapitated at hourly interval between 1500 - 1800h on day 3. The number of animals used is shown in brackets.

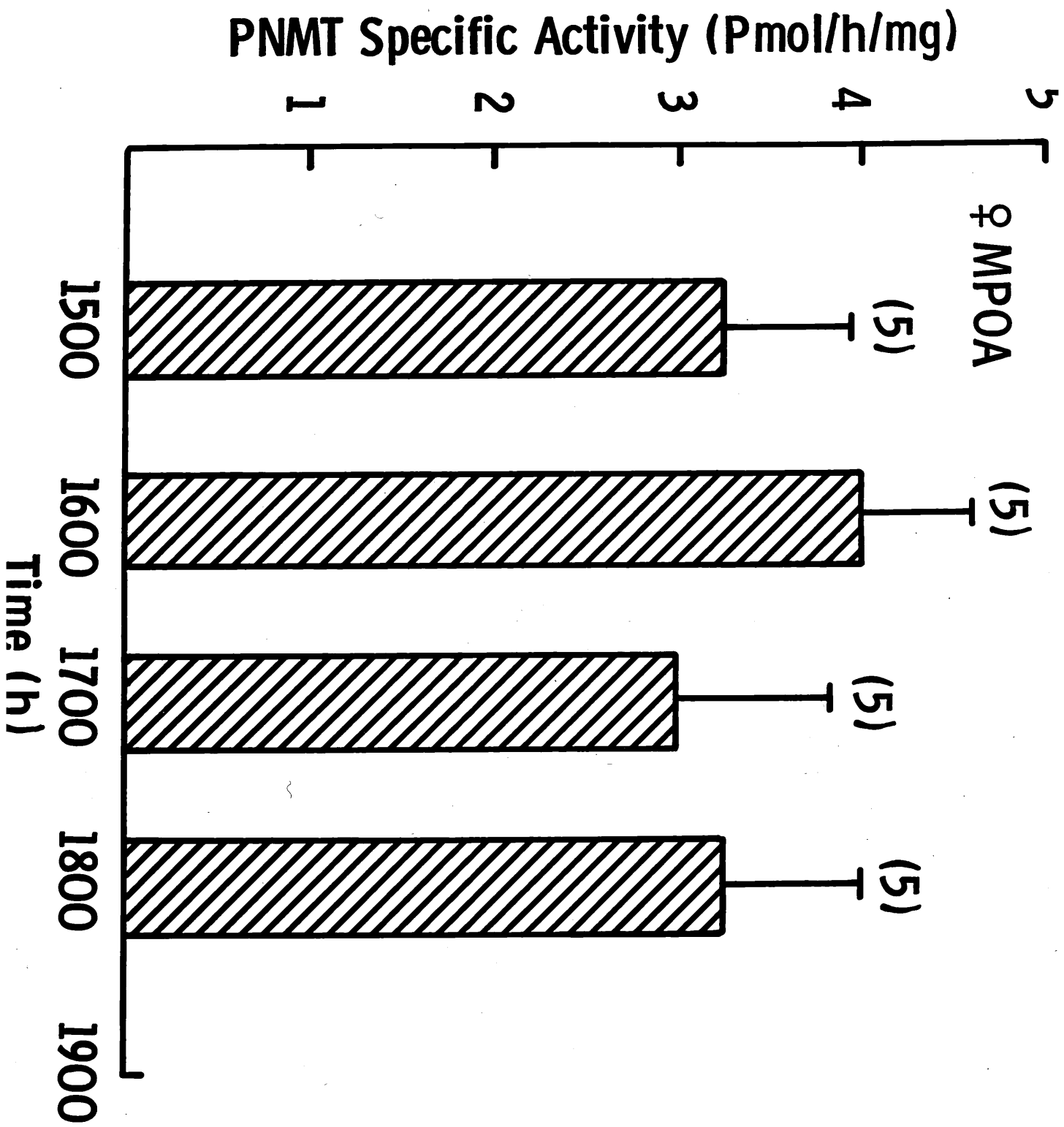
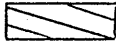



Fig.20. Mean specific activity ( $\pm$ S.E.M.) of phenylethanolamine-N-methyl transferase (PNMT) in the mediobasal hypothalamus of 22-day-old male rats injected with either oil (  ) or oestradiol benzoate (  ) at 1200h on experimental day 1 and decapitated at 1500h, 1600h or 1700h on day 3. The number of animals used is shown in brackets.

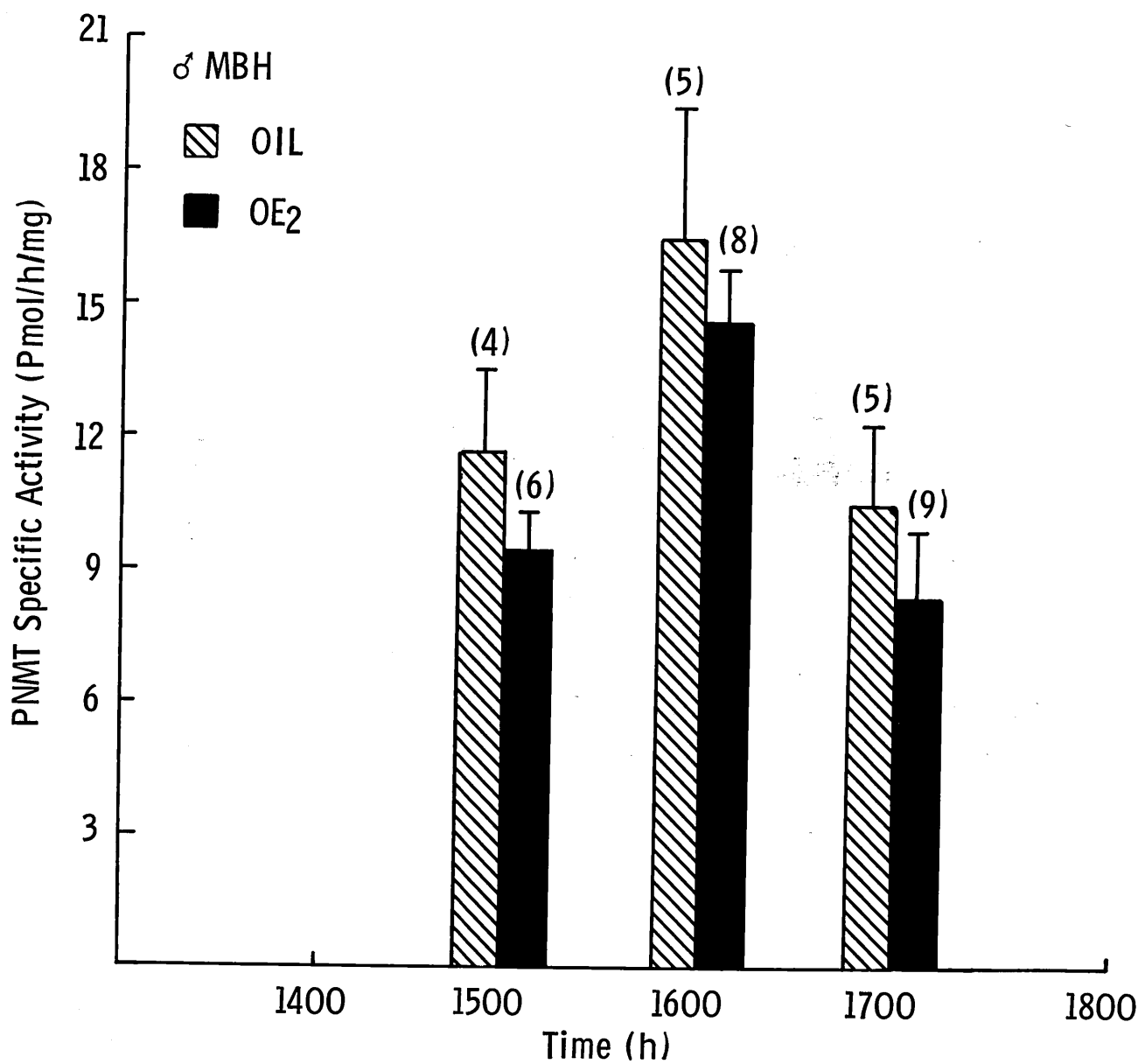


Table VII.Mean specific activities ( $\pm$  SEM) of phenylethanolamine-N-methyl transferase (Pmol PNMT/h/mg protein) in the mediobasal hypothalamus of groups of 16 and 22 day old female rats. The animals were injected (s.c.) with either OE<sub>2</sub> or oil at 1200h on day 1 and killed in groups at hourly intervals during the afternoon of day 3. The number of animals used is shown in brackets.c : a (P<0.01); c : e (P<0.01)

Days of age	Treatment	Mean specific activities of PNMT on experimental Day 3				
		<u>1400h</u>	<u>1500h</u>	<u>1600h</u>	<u>1700h</u>	<u>1800h</u>
16	OE <sub>2</sub>	5.58 $\pm$ 1.03 (n=5)	3.54 $\pm$ 0.71 (n=6)	5.37 $\pm$ 1.02 (n=5)	4.27 $\pm$ 1.01 (n=5)	-
22	oil	-	3.55 $\pm$ 0.86 (n=5)	3.52 $\pm$ 0.36 (n=5)	5.56 $\pm$ 2.33 (n=5)	5.55 $\pm$ 1.08 (n=4)
22	OE <sub>2</sub>	5.49 $\pm$ 1.28 <sup>a</sup> (n=6)	9.04 $\pm$ 1.66 <sup>b</sup> (n=10)	12.25 $\pm$ 1.38 <sup>c</sup> (n=10)	9.87 $\pm$ 1.13 <sup>d</sup> (n=10)	7.39 $\pm$ 1.19 <sup>e</sup> (n=9)

Fig.21. Mean specific activity ( $\pm$ S.E.M.) of phenylethanolamine-N-methyl transferase (PNMT) in the mediobasal hypothalamus of 22-day-old female rats injected with 1 $\mu$ g oestradiol benzoate at 1200h on experimental day 1. Groups of animals were decapitated at 4-hourly intervals between 1200h on experimental day 2, and 2000h on day 3. The number of animals used is shown in brackets. Horizontal white and black bars on the ordinate line indicates hours of light and dark respectively.

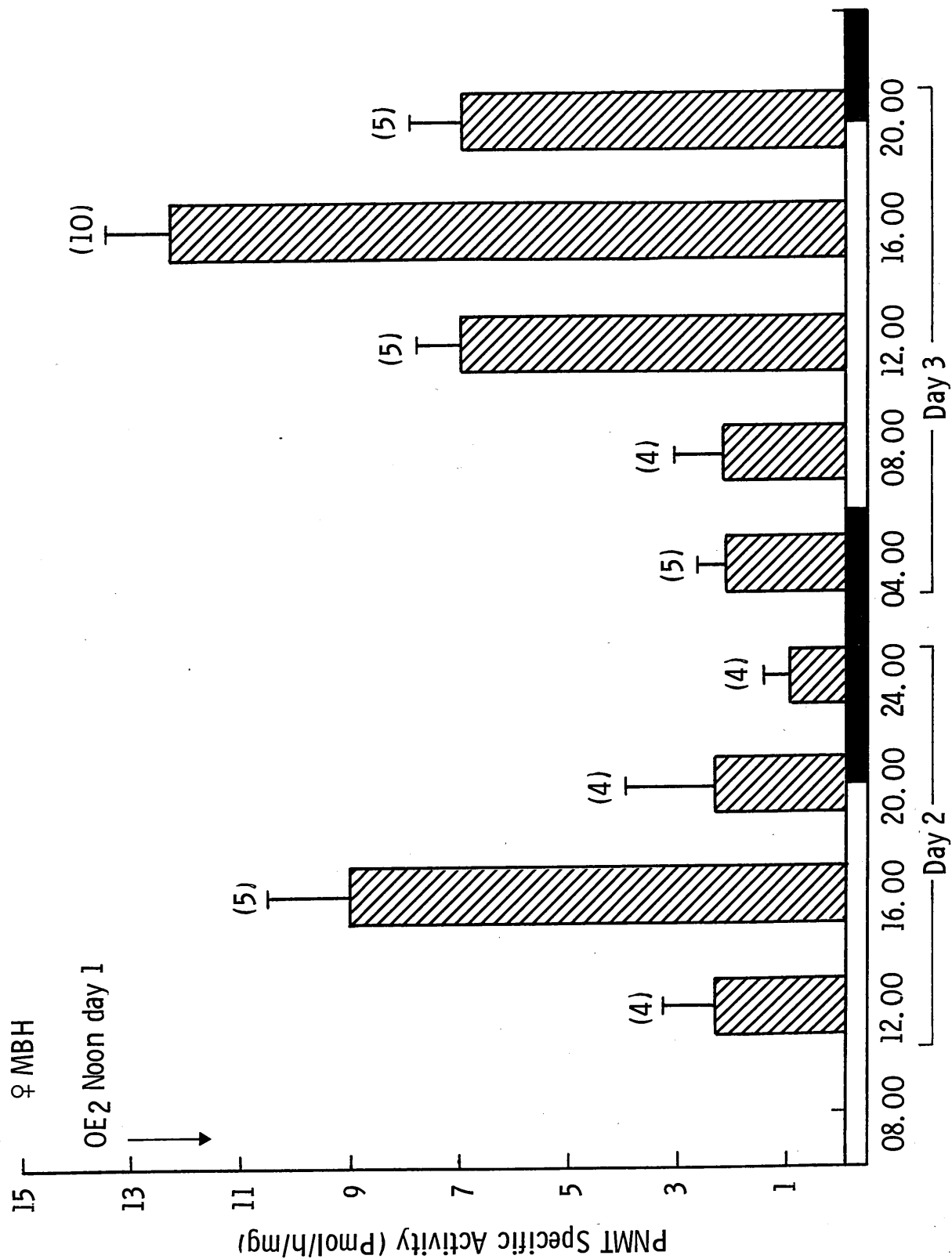
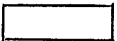

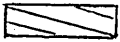
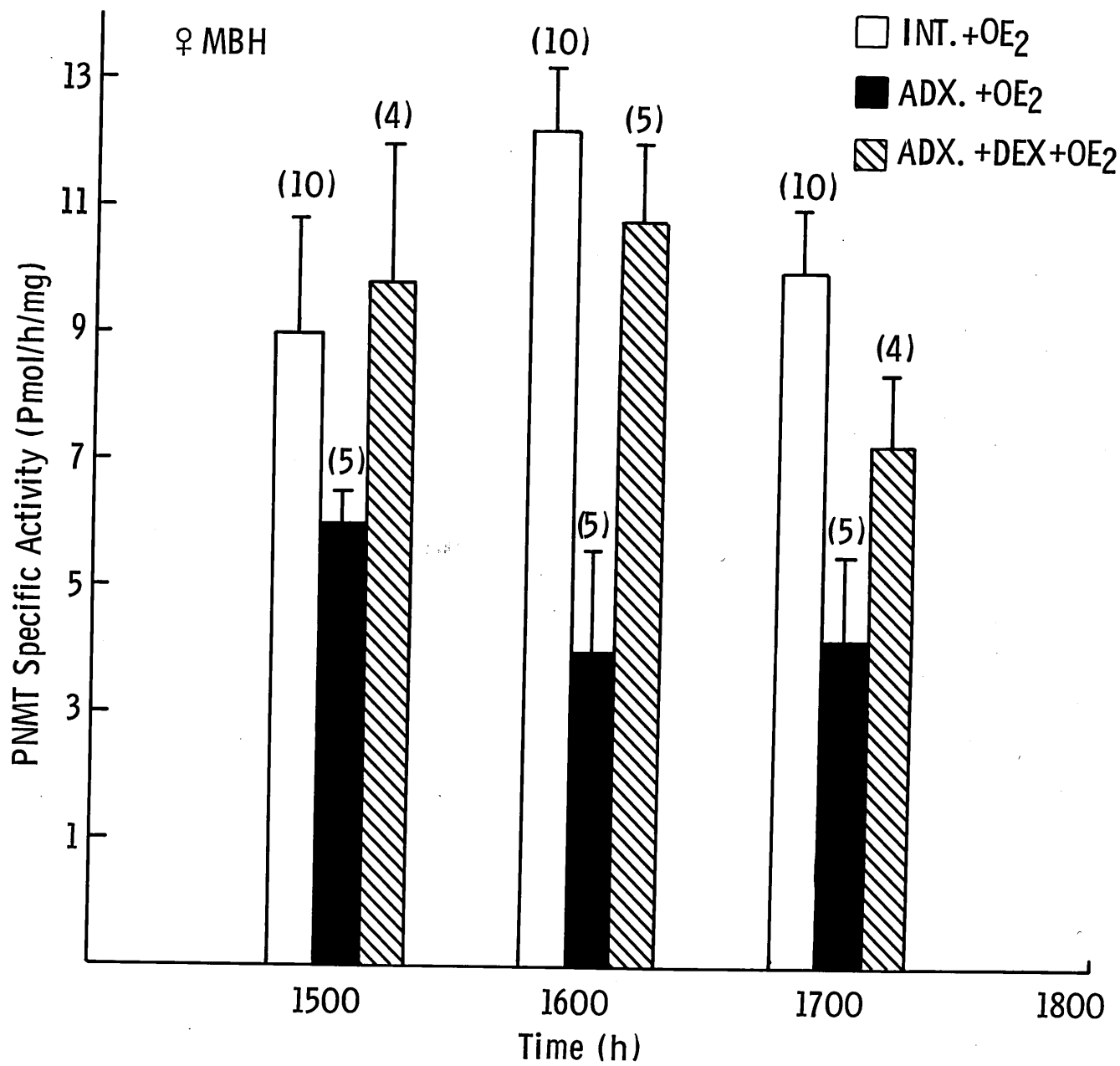




Fig.22. Mean specific activity ( $\pm$ S.E.M.) of phenylethanolamine-N-methyl transferase (PNMT) in the mediobasal hypothalamus of groups of 22-day-old oestrogen-treated female rats with intact adrenals (  ) or adrenalectomized (  ) and adrenalectomized and treated with dexamethasone (  ).

The number of animals used is shown in brackets.

\*\* 1600h : ADX + OE<sub>2</sub> vs ADX + DEX + OE<sub>2</sub> (P<0.01)



## Discussion:

These experiments demonstrate that there is a significant increase ( $P < 0.01$ ) in the mean activities of PNMT in the MBH of both 22-day-old oestrogen-treated male and female rats at 1600h on day 3 (Fig.18). Such an effect was not seen in the MPOA of these female rats (Fig.19). The adrenaline concentrations in the MPOA and MBH of similarly treated rats were basal; with the exception of MBH of female rats which showed a significantly ( $P < 0.01$ ) higher increase in adrenaline concentration at 1600h (see Chapter 4, Figs. 16 and 17).

Interestingly, the MBH (a discrete area of the rat brain associated with the release of GnRH) of oestrogen-treated 22-day-old female rats showed both an increase in PNMT activity and an increase in adrenaline concentration at 1600h on day 3. In contrast, the MPOA (a discrete area of the rat brain associated with the synthesis of GnRH) of these rats was without such an effect. This observation of an increase in adrenaline concentration is confirmation of an earlier study in which the MBH of oestrogen-treated acutely ovariectomized rats at the expected proestrus showed an increase in adrenaline concentration and, furthermore a higher adrenaline turnover during the neural critical period (Mackinnon et al., 1985). Likewise, the MPOA of these rats was devoid of such a response. Using antibodies to PNMT, it was demonstrated that cell bodies of adrenergic neurones, the axons of which project to the MPOA and MBH, are situated in the lateral reticular nucleus of the medulla (Hökfelt et al., 1980). Moreover, employing both autoradiographic and immunohistochemical techniques, it was shown that both tritium labelled oestrogen and the enzyme dopamine- $\beta$ -hydroxylase were localised in the cells of the same region of the medulla (Sar & Stumpf, 1981). Based on these observations it can be proposed that oestrogen might act indirectly, via an adrenergic

pathway on axon terminals of GnRH synthesizing and possibly TRH synthesizing neurones. In support of this suggestion is the observation that oestrogen does not concentrate in the cell bodies of GnRH immunoreactive neurones (Shivers et al., 1983). Furthermore, a recent study has indicated that increased adrenergic activity seen in the MBH of proestrous rats was related not so much to the arcuate nucleus as to the median eminence (Sheaves et al., 1984).

In contrast to oestrogen-treated female rats, male rats showed a significantly higher activity of PNMT in the MBH, while there was no increase in adrenaline concentration in response to an oestrogen stimulus. The absence of an increase in adrenaline concentration might be accounted for by an increase in its release or by enhanced monamine oxidase and/or catechol-o-methyl transferase activities at the MBH level. Whether these enzymes involved with monoamine oxidase metabolism are sexually differentiated is not known. However, it could be argued that a continuous release of adrenaline into the hypothalamo-hypophyseal portal vessels could result in baseline levels of adrenaline in the MBH of oestrogen-treated male rats. In similarly treated female rats an increase in concentration of adrenaline in the MBH might be due to its release having been inhibited. If this were the case then it is possible that central adrenaline might be operative at the level of the anterior pituitary. Possibly the presence of an increased level of adrenaline in the pituitary might inhibit the output of prolactin and LH in males. Furthermore, its possible disinhibition in females might cause the output of these hormones. One piece of evidence which supports this argument comes from a previous study in which with the use of high performance liquid chromatography and electrochemical detection, Johnston and his co-workers demonstrated that adrenaline concentrations in the portal blood were significantly higher than peripheral levels of adrenaline both in intact and adrenalectomized

adult male rats (Johnston et al., 1983). With respect to adult females however, intensive studies of adrenaline concentrations in portal blood in this laboratory have failed to show any change in the levels during the oestrous cycle. At present these portal blood results reported by Johnston and co-workers are difficult to interpret. However, the results pertaining to the measurements of adrenaline in the portal blood from our laboratory suggest that central adrenaline might be operative at the MBH level rather than at the pituitary. If this were the case then it is likely that the adrenergic event in MBH represents a paracrine control of nerve terminals of releasing hormones (possibly TRH and GnRH), since there is no evidence to date for axo-axonal synaptic contacts between catecholaminergic and releasing-hormone neurones.

It is generally accepted that the removal of an inhibitory effect of endogenous opioids allows an enhanced turnover of catecholamine (NA & A) which apparently influences the release of GnRH (Kalra & Kalra, 1983). In the light of this observation, one possible interpretation for the differences in adrenaline concentration present in the MBH of oestrogen-treated infant male and female rats could be that disinhibitory effect of the endogenous opioids was only expressed in females. The mechanism by which endogenous opioids exert their inhibitory/disinhibitory effect on catecholamines is still unclear.

It was of interest to ascertain whether the increase in PNMT activity in the male rats was due to the presence of exogenous oestrogen. Using the same experimental regime further studies demonstrated that oil-treated 22-day-old males showed an increase in PNMT activity in the MBH similar to that seen in oestrogen-treated male rats (Fig.20). This suggests that an increase in PNMT activity is not due to a response to exogenous

oestrogen. One possibility for this oil-treated rats could be that these have high levels of endogenous oestrogen due to aromatization of androgens. The oestrogen possibly in turn influences the cell bodies of PNMT containing neurones which project their axons to the MBH. Consequently, the oestrogen-induced newly synthesized PNMT might be axonally transported to the MBH, therefore resulting in an increase in its concentration. Possibly the PNMT containing neurones are responding at maximum level to its endogenous oestrogen system(s) and hence, exogenous oestrogen does not seem to have any effect in further increasing the PNMT activity.

Since a close temporal relationship seems to exist in the oestrogen-treated infant female rats between increased adrenaline concentration in the MBH and a massive output of gonadotrophins (prolactin and LH) 2h later, it is possible that it might be dependant on an oestrogen-enhanced circadian influence on the adrenaline synthesizing enzyme. Subsequent measurements of oestrogen-stimulated PNMT activity in groups of rats decapitated at 4-hourly intervals from 1200h on day 2 to 2400h on day 3, showed that this was indeed the case, peak levels being attained at about 1600h with a nadir in the early hours of the morning (Fig.21). There was, however, no evidence of an increase in PNMT activity in oestrogen-treated 16-day-old females, oil-treated 22-day-old females (Table V), or in adrenalectomized oestrogen-treated 22-day-old females (Fig.22) at 1600h. These observations suggest that PNMT activity is inducible by both oestrogen and glucocorticoids since the presence of oestrogen and dexamethasone reinstated the enhanced levels of PNMT activity (Table V, Fig.22). These results are in agreement with other workers who found that glucocorticoids modulate both adrenal and brain PNMT activities (Wurtman, 1966; Pohorecky et al., 1969; Ciaranello, 1978). However, there is no evidence in the literature that brain PNMT is inducible by oestrogen. In a study pertaining

to peripheral PNMT, however, it was demonstrated that repeated injection (s.c.) of oestradiol benzoate during late pregnancy in rats enhanced PNMT activity (Raja-Bukhari et al., 1976). This result is in agreement with the present finding that a single injection (s.c.) of oestrogen in 22-day-old female rats results in an increase in PNMT activity.

In contrast to 22-day-old oestrogen-treated females, 16-day-old oestrogen-treated female rats did not show an increase in PNMT activity in the MBH at anytime on day 3 which was concomitantly associated with basal levels of adrenaline concentrations. This suggests that an increase in adrenaline concentration in the MBH of oestrogen-treated female rats is closely related to the age of the animal. It could be argued that a failure of 16-day-old females to respond to an oestrogen stimulus was prevented by the presence of  $\alpha$ -foeto-protein ( $\alpha$ FP) (see Chapter 3). However, an injection (s.c.) of stilboestrol which has a low affinity for  $\alpha$ FP was also without effect. It is possible, therefore, that a failure of the surge output of prolactin and LH in these animals was due to the absence of an increased concentration of adrenaline in the MBH.

In conclusion, present studies of the oestrogen-treated 22-day-old female rat suggest that there is a clear circadian rhythm in the MBH which is oestrogen and possible glucocorticoid dependant. Furthermore, an increase in PNMT activity in the MBH is paralleled by an increase in adrenaline concentration.

### GENERAL DISCUSSION AND CONCLUDING PROPOSITIONS

This chapter attempts to highlight and correlate the main issues concerned with the oestrogen treated immature rats of 16-25 day-old (the 'infant rat' model') which have arisen during the course of this work. Namely, the increase in adrenaline concentration in the MBH which invariably takes place 2h (1600h) before the oestrogen stimulated prolactin and LH surge outputs in 22-day-old female rats, but not in younger females or males at any age. This is in synchrony with the increase in activity of its synthesizing enzyme, PNMT. Furthermore, the increase in PNMT activity in the MBH of 22 day-old female rats is sensitive to the presence of steroids and shows a circadian rhythm with a peak value occurring at 1600h and a nadir at 2400h.

The immediate question which arises pertains to the choice of the oestrogen treated infant rat as an experimental model for the study presented in this thesis. It was selected for three reasons. One was that an injection of oestradiol benzoate ( $OE_2$ ) on experimental day 1 at as early an age as 21 days caused a surge output of prolactin at 1800h of days 2 and 3; while a surge of LH occurred only at 1800h on day 3 (Puig-Duran & Mackinnon, 1978a,b). Similar treatment of younger females and males at any age had no such effect. Secondly, it came from the studies performed both in this laboratory and elsewhere which showed that adult proestrus rats or long-term oestrogen treated ovariectomized rats when injected (i.p.) with SKF64139 (an inhibitor of central adrenaline synthesis) blocked the LH surge and consequent ovulation (Coen & Mackinnon, 1981; Coen et al., 1982; Crowley & Casterly, 1981). In a third study it was found that a marked increase in adrenaline concentration and its turnover occurred both in the MPOA



and MBH of adult proestrus rats during the proestrous 'critical period', prior to the preovulatory LH surge (Mackinnon et al., 1983). Using acutely ovariectomized adult rats it was found that the increase in adrenergic activity in the MBH, but not apparently that obtained in the MPOA, was oestrogen dependant (Mackinnon et al., 1985). It was suggested from these observations that oestrogen stimulated adrenergic activity in the MBH of adult rats might be concerned with the release of GnRH from their nerve terminals and an ensuing massive output of LH. It was therefore of interest to investigate the development of central adrenergic systems in the MBH concerned with the ontogeny and sexually differentiated output of LH in response to an oestrogen (OE<sub>2</sub>) stimulus. A possible approach to this problem was to measure adrenaline concentration and the activity of its synthesizing enzyme (PNMT) present in the MPOA and MBH of OE<sub>2</sub> treated 16- and 22-day-old females and 22-day-old males at relevant times, prior to surge outputs of prolactin and LH. This approach was undertaken throughout the present study.

The techniques which have been used throughout the present work were found to be valid and reliable. The rabbit antibody to ovine LH used in the RIA of LH cross reacts avidly with the rat LH and furthermore, shows negligible cross reactivity against FSH and TSH (Niswender et al., 1968). The observation that inter- and intra-assay coefficients of variation are within the acceptable range, further supports the validity of this assay (see Section 'A' of Chapter 2).

Due to consistency in the occurrence of landmarks for the location of the MPOA and MBH the use of micropunches as described by Palkovits (1973) was found to be highly reliable (see Section 'B' of Chapter 2).

The low values for inter- and intra-assay coefficients of variation and the authentic standards made up in the tissue supernatant and used

in parallel to unknown samples for the radioenzymatic assays of both adrenaline and PNMT support the validity and reliability of these assays (see Sections 'C' and 'D' of Chapter 2).

The Lowry protein assay was used to measure small protein concentrations in the MPOA and MBH of the infant rat. This assay was highly reliable, and here again low values of inter- and intra-assay coefficients of variation were obtained. Furthermore, each assay included BSA standards in parallel to unknown samples (see Appendix).

The preliminary experiments as described in Chapter 3 were undertaken to confirm earlier findings from this laboratory with respect to the infant rat model. In 21-, or 22-day-old female rats marked increases in the concentrations of serum LH were obtained at a well defined time on day 3 (1800h), 54h after injection of oestrogen; while similarly treated 16 day-old females and males at any age showed no such effect (see Chapter 3). These results confirm the findings from this laboratory and extend earlier observations on the role of oestrogen in the facilitation of the release of LH in the serum of the immature female rat (Ramirez & Sawyer, 1965; Caligaris et al., 1972). A marked, but short-lived increase in adrenaline concentration in the MBH of oestrogen treated 22-day-old female rats was observed at 1600h on either day 2 or day 3, 2h before the surge output of gonadotrophins (prolactin with or without LH). But oestrogen treated 16-day-old females and 22-day-old males did not show such a response on day 3. Furthermore, in contrast to the results that were obtained in the MBH, the MPOA of oestrogen treated 22 day-old females did not show an increase in the adrenaline concentration (see Chapter 4). The increased levels of adrenaline in the MBH of oestrogen treated 22-day-old female rats, are much higher than those seen in the MBH of proestrus or

acutely ovariectomized adult rats prior to the surge outputs of their gonadotrophins (Mackinnon et al., 1983; 1985). One possibility for this difference could be the way that MBH has been punched out. Since an earlier study revealed that a much higher proportion of adrenaline was present in the lateral and medial median eminence than in the arcuate nucleus (Sheaves et al., 1984); the MBH of OE<sub>2</sub> treated 16- and 22-day-old females or 22-day-old males was punched out in such a manner as to include a large proportion of ME, but only small amount of arcuate nucleus. The MBH is located outside the blood-brain barrier, so it could be argued that the increase in adrenaline concentration which occurs in the MBH of oestrogen treated 22-day-old rats is not of central origin; but instead possibly reflects an increase in the output of adrenaline from the adrenals. However, the MBH of adrenalectomized-dexamethasone-oestrogen treated 22-day-old female rats showed an increase in adrenaline on experimental day 3, similar to those seen in the oestrogen treated intact rats (Personal Communication, A. Melcher). This finding suggests that an increase in adrenaline concentration in the MBH which has been measured in the present study does not reflect release from adrenals.

The important question which arises is whether or not there is a physiological role for the increase in central adrenaline concentration which occurs consistently not only in the MBH of proestrus or acutely ovariectomized oestrogen treated rats, but also in the oestrogen treated 22 day-old female rats. It has been suggested that central adrenaline in the MBH might be temporally related to the output of gonadotrophins. Whether it is temporally related to prolactin alone still remains to be seen. However, evidence that adrenaline might be a potent catecholamine in stimulating the output of prolactin came from a study in which steel cannulae were bilaterally implanted into

the MBH of adult male rats (Day et al., 1982). This study showed that adrenaline had the greatest effect in evoking prolactin release following the bilateral micro-injections of DA, NA or A into the MBH of these rats. Earlier studies from this laboratory and elsewhere demonstrated that a central adrenaline synthesis inhibitor (SKF64139) blocked the surge output of LH (Coen & Mackinnon, 1981; Coen et al., 1982; Crowley et al., 1982). However, the use of this inhibitor is open to criticism. The observation that it possesses  $\alpha$ -receptor antagonistic properties (Toomey et al., 1981) and furthermore, inhibits monoamine oxidase activity (an enzyme responsible for metabolising catecholamines) (Fuller & Hemrick-Luecke 1983). It is possible therefore that blockade of the output of gonadotrophin by the use of SKF64139 might possibly be due to its  $\alpha$ -receptor antagonistic properties; since catecholamines (NA & A) which might be involved in the regulation of the surge output of gonadotrophin are generally considered to mediate their response through these receptors (Everett et al., 1949; Sawyer et al., 1950; Drouva et al., 1982). This seemed to be the case since an almost complete reduction in central adrenaline concentration in the MBH of adult proestrus rats using a more recently synthesized drug was commensurate with an LH surge and ovulation (Sheaves et al., 1985b). This drug 8,9-dichloro-2,3,4,5-tetra-hydro-1H-2-benzazepine (LY134046) has a much lower affinity for  $\alpha$ -receptors than SKF64139 (Fuller et al., 1981). Such evidence reduces the possibility of a role for central adrenaline in the output of LH, but it still remains to be seen whether or not this obtains in oestrogen treated infant rats. Whether the increase in MBH adrenaline is temporally related to the output of any other hormones such as TSH (Brown-Grant et al., 1977) or ACTH (Buckingham et al., 1978) is not known. For that matter neither is it

known whether the infant rat model shows surge outputs of pituitary hormones other than those that have been measured (prolactin and LH) in this laboratory. To investigate this possibility the anterior and posterior pituitary hormones which are known to be released in response to an oestrogen stimulus should be measured in saline injected (control group) and in LY134046 treated animals. At present, however, it can only be said that an increase in adrenaline concentrations in the MBH of oestrogen treated 22-day-old female rat are temporally related to the output of gonadotrophins. The hypothesis that an increase in adrenaline concentration in the MBH is possibly concerned with the output of gonadotrophins finds support in the present observations in which oestrogen stimulated 16-day-old females and 22-day-old males failed to show an increase in either adrenaline concentration in the MBH or a subsequent outpouring of gonadotrophins. Since  $\alpha$ -foeto protein ( $\alpha$ -FP) concentrations in the blood decline steadily from birth to 21 days of age, it can be argued that the failure to show a gonadotrophin response to an oestrogen stimulus in 16-day-old rats was due to the presence of this oestrogen binding protein (Nunez et al., 1971). However, in rats treated with diethylstilboestrol (a synthetic oestrogen which does not bind avidly to  $\alpha$ -FP) the gonadotrophin positive feedback response to synthetic oestrogen was also absent (Puig-Duran & Mackinnon, 1978b). A possible cause for this failure to show a surge output of gonadotrophins in response to synthetic oestrogen, could be the absence of an increase in adrenaline concentration in the MBH of these rats.

The immediate question which arises as to how this increase in adrenaline concentration in the MBH of our rat model regulates the output of hormones such as prolactin and LH and possibly ACTH and TSH. It has been discussed and, furthermore suggested that an increase in

adrenaline might regulate the output of releasing hormones such as GnRH, CRH or TRH into the hypothalamo-hypophyseal portal vessels possibly through a paracrine like effect (see the discussion section of Chapter 5). Whether the measurements of an increase in adrenaline concentration in the MBH of oestrogen treated 22-day-old female rats represents an accumulation of adrenaline within the nerve terminals or an increased output of adrenaline into the extravascular space or portal vessels is not known. A further question which arises as to how this increase in adrenaline concentration occurs. Measurements of PNMT activity in the MBH of oestrogen treated 16- and 22-day-old female showed that this might be the possible candidate involved with the increase in MBH adrenaline concentration, since only 22-day-old animals showed an increase in PNMT at 1600h on day 3 in parallel to the increase in adrenaline (see Chapter 4 & 5). Furthermore, it has been shown previously that both in the adrenals and the brain, adrenaline concentrations vary directly with PNMT activity (Wurtman & Axelrod, 1966; Moore & Phillipson, 1975). What determines the increase in PNMT activity and consequently an increase in adrenaline in the 22-day-old females but not in 16-day-old females in response to an oestrogen stimulus is not known. However, a possible answer to this question comes from previous studies on opioids. Several studies have implied that the removal of an inhibitory effect of endogenous opioids allows an enhanced turnover of catecholamines (NA & A) which apparently influence the release of GnRH (Kalra, 1982; Kalra & Kalra, 1983). It is possible that an increase in adrenaline concentration (presumably reflecting a higher turnover) (Hiemke et al., 1985) was due to the disinhibitory effect of the endogenous opioids on adrenaline neurones which might only be expressed in oestrogen treated 22-day-old females. Contrary to this concept that increased adrenaline concen-

trations vary directly with PNMT activity was the observation that OE<sub>2</sub> treated 22-day-old male rats also showed an increase in PNMT activity at 1600h (day 3) similar to that shown by the females; although no increases were found in adrenaline concentration in the MBH in response to oestrogen (see Chapter 4 & 5). PNMT was extracted from the MBH of either male or female rats and its activity measured under optimum conditions, it is possible therefore that there may be physiological differences in the enzyme activity of that found in males. The differences may be due to a number of factors such as the ratio of S-adenosylmethionine to homocysteine (Deguchi & Barchas, 1970), the ionic strength of the enzyme environment (Cubeddu & Vargas, 1977). Whether one or both these factors regulating PNMT activity are sexually differentiated is not known. This increase in PNMT activity in the MBH of 22-day-old male on day 3 is not only shown in oestrogen treated animals, but also in oil-treated animals (control group) (see Fig.20, Chapter 5). It has been suggested that the increase in PNMT activity in the control group, which was similar to that found in oestrogen treated rats, might be due to the presence of endogenous androgens and their metabolites as discussed previously (see Chapter 5). However, in the MBH of 22-day-old female rat an increase in PNMT activity at 1600h on day 3 was not seen in the absence of an oestrogen stimulus (see Table VII Chapter 5). Furthermore, it has been observed that oil-treated 22-day-old females also showed baseline levels of adrenaline in the MBH in parallel to the low PNMT activity (Table VI, Chapter 4. However, the MBH of oil treated acutely ovariectomized adult rats showed low adrenergic activity (1430h - 1630h); this is the time when it was found to be much higher in intact proestrus and oestrogen treated acutely ovariectomized rats (Mackinnon et al., 1985). How does oestrogen regulate PNMT activity requires investigation. One possibility could be that oestrogen stimulates the genomic mechanisms of PNMT

synthesis at the cell body level and the PNMT synthesized consequently is axonally transported to their terminals in the MBH. Support for this suggestion came from a study in which it was demonstrated that tritiated oestradiol concentrated in the nuclei of catecholaminergic neurones in the brain stem (Sar & Stumpf, 1981). This implies that genomic activation induced by oestrogen might be operative at the catecholaminergic neurone cell body level. Furthermore, a retrograde tracing study has clearly shown that a dye (Fast blue) when injected into the MPOA of rats was histologically verified to be in the 3rd ventricle (Saper et al., 1983). This dye was later detected in PNMT-positive cell bodies located in the C<sub>1</sub> cell groups of the medulla oblongata. This provides evidence for the existence of adrenergic neurones projecting their axons from this cell group to the hypothalamus. Further support came from a study of the measurements of brain stem PNMT activity in adult rats at 4-hourly intervals over 24h period (Turner et al., 1981). This study showed the presence of a clear circadian rhythm which reached peak levels at 1500h. The study presented in this thesis (see Fig. 21, Chapter 5) has also shown that a circadian rhythm of PNMT activity in the MBH exists in the oestrogen treated 22-day-old females, with a peak occurring at 1600h and a nadir at 2400h. The one hour lag period between the peak of PNMT activity occurring in the brain stem and that of MBH might possibly represent the time taken by the fast exoplasmic flow responsible for transporting this enzyme from the cell bodies to their terminals in the MBH. Such a process has been shown to be operative at the rate of 100mm per day (Kapeller & Mayor, 1967). Whether the existence of PNMT circadian rhythms in the brain stem of oestrogen treated 22-day-old rats is also present, similar to that of adult rats, still remains to be seen. A study was performed by Brownstein et al., 1976 in which PNMT activity measured in the median



eminence and arcuate nucleus of normal rats or in that of long-term rats in which the hypothalamus was completely deafferentated. This study showed that only a partial decrease in PNMT activity occurred after total hypothalamic deafferentation. Brownstein and co-workers postulated that the existence of adrenergic neurone cell bodies might be present in the hypothalamus in addition to that present in the brain stem. If this were the case then it is possible that oestrogen might induce genomic activation in the adrenergic cell bodies at the hypothalamic level as well.

The increase in PNMT activity in the MBH of 22-day-old females at 1600h on day 3 is dependant not only on an increase in oestrogen concentrations in the plasma, but also on the presence of plasma glucocorticoids. Since it is only adrenalectomized steroids (oestrogen and dexamethasone) treated animals that showed an increase in PNMT activity similar to those of oestrogen treated intact rats; while adrenalectomized-oestrogen treated animals (control group) did not show such a response (see Fig.22, Chapter 5). How these corticosteroids regulate the PNMT activity is not fully understood. One possibility could be that corticosteroids are operative in inhibiting the proteolysis of this enzyme . At present there is no direct evidence available for the regulatory role of corticosteroids in inhibiting the proteolysis of an enzyme in vivo. However, thermal denaturation studies of the PNMT extracted from adrenals of either normal or hypophysectomized rats showed that the PNMT activity of hypophysectomized animals was much more vulnerable than that of normal rats (Ciaranello et al., 1978). Furthermore, the presence of ACTH or dexamethasone in the plasma of hypophysectomized rats reduced the vulnerability to thermal denaturation in vitro. This difference in PNMT activity, as measured under optimum conditions after heat denaturation at 50°C in the normal and hypophys-

ectomized animals thought to be due to the presence of a stabilizing factor, S-adenosylmethionine (SAM); since the stabilizing factor was only present in the PNMT extracted from normal rats. Ciaranello and his co-workers postulated that an increased susceptibility of PNMT to heat denaturation in vitro possibly reflects the increased susceptibility of this enzyme to intracellular proteolysis in vivo. It is possible that endogenous corticosteroids induce the binding of SAM to the PNMT. Binding of SAM might alter the enzyme conformation in such a manner that it is no longer preferred substrate for proteolysis. In contrast to the present finding that glucocorticoids stimulate PNMT activity in the MBH of oestrogen treated 22-day-old female rats at 1600h (day 3), an injection (s.c.) of corticosterone failed to elevate brainstem PNMT activity within 1h compared to either vehicle injected or uninjected controls (Turner et al., 1981). However, the long-term effect of glucocorticoids in enhancing the PNMT activity cannot be ruled out since adrenalectomized-dexamethasone-oestrogen treated animals have shown a significant rise ( $P < 0.01$ ) at 1600h compared to adrenalectomized-oestrogen treated animals (see Fig. 22, Chapter 5). Whether or not the onset of PNMT circadian rhythms are stimulated by corticosteroids in the oestrogen treated rats still remains to be seen. However, a possible effect of glucocorticoids in enhancing the PNMT activity in the MBH and consequently adrenaline concentration in the MBH of oestrogen treated rats finds support from the present observations. Oestrogen treated 16-day-old females in which apparently corticosteroid rhythms have not yet been established (Ader, 1969; Campbell & Ramaley, 1974) did not show an increase in PNMT activity, nor did they show an increase in adrenaline (see Chapters 4 & 5). Further work is required to investigate changes in the concentration of serum corticosteroids in oestrogen treated 16-

and 22-day-old remale rats. In adult rats corticosteroid rhythms appear to be related to, though not necessarily dependant on circadian rhythms of hypothalamic CRH (Seiden & Brodish, 1972) and serum ACTH concentrations (Buckingham et al., 1978). Since a surge output of ACTH at proestrus in adult rats occurs in synchrony with the pre-ovulatory surge of gonadotrophins (Buckingham et al., 1978). The possibility exists that increase in PNMT activity and adrenaline concentrations in the MBH which precede by 2h a massive output of gonadotrophins (prolactin and LH) might be more closely related to ACTH output.

In conclusion, a temporal relationship (not necessarily specific) exists between increased adrenaline concentrations in the MBH of oestrogen treated 22 day-old female rats and a surge output of gonadotrophins (prolactin with or without LH) 2h later. This relationship apparently depends on an oestrogen and possibly glucocorticoids stimulated circadian rhythm of PNMT activity. Failure of an increase in the activity of PNMT and concentration of adrenaline in the MBH of oestrogen treated 16 day-old females; and furthermore, disruption of the synchrony between increased PNMT activity and adrenaline concentration in the MBH of similarly treated males might be responsible for the absence of an outpouring of gonadotrophins (prolactin and LH).

Ader, R. (1969)

Early experiences accelerate maturation of the 24-hour adrenocortical rhythm.

Science 163, 1225-1226

Ahren, K., Dekel, N., Hamberger, L., Hillensjo, T., Hultborn, R., Magnusson, C., and Tsafurum, A. (1978)

Metabolism and morphological changes produced by gonadotrophins and cyclic AMP in the oocyte and cumulus oophorus of preovulatory rat follicle.

Ann. Biol. Anim. Biochem. Biophys. 18, 409

Aiyer, M.S. & Fink, G. (1974)

The role of sex-steroid hormones in modulating the responsiveness of the anterior pituitary gland to luteinizing hormone-releasing factor in the female rat.

J. Endocr. 62, 533-572

Aiyer, M.S., Fink, G. & Greig, F. (1974a)

Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor during the oestrous cycle of the rat.

J. Endocr. 60, 47-64

Aiyer, M.S., Chiappa, S.A. & Fink, G. (1974b)

A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat.

J. Endocr. 62, 573-588

Aiyer, M.S., Sood, M.C. & Brown-Grant (1976)

Pituitary response to exogenous luteinizing hormone releasing factor in steroid-treated gonadectomized rats.

J. Endocr. 69, 255-262

Ajika, K. & Hökfelt, T. (1973)

Ultrastructural identification of catecholamine neurones in the hypothalamic periventricular-arcuate nucleus-median eminence complex with special reference to quantitative aspects.

Brain Res. 57, 97-117

Ajika, K. & Hökfelt, T. (1975)

Projections to the median eminence and the arcuate nucleus with special reference to monoamine systems : effects of lesions.

Cell Tissue Res. 158, 15-35

Alp, P.R., Zammit, V.A. & Newsholme, E.A. (1976)

Activities and some properties of adenylate cyclase and phosphodiesterase in muscle liver and nervous tissues from vertebrates and invertebrates in relation to the control of the concentration of adenosine 3:5-cyclic monophosphate.

Biochem.J. 154, 689-700

Appel, E., Bayer, P., Hajdu, P., Palm, D., Schofer, J. & Uihlein, M. (1981)

Determination of plasma catecholamines by means of radioenzymatic labelling and high pressure liquid chromatographic separation.

Nauman-Schmiedeberg's Arch. Pharmacol. 315, 233-239

Arch, J.R.S. & Newsholme, E.A. (1976)

Activities and some properties of adenylate cyclase and phosphodiesterase in muscle liver and nervous tissues from vertebrates and invertebrates in relation to the control of concentration of adenosine 3:5-cyclic monophosphate.

Biochem. J. 158, 603-622

Axelrod, J. & Tomchick, R. (1958)

Enzymatic O-methylation of epinephrine and other catechols.

J. Biol. Chem. 232, 702-705

Axelrod, J. (1962)

Purification and properties of phenylethanolamine-N-methyl transferase.

J. Biol. Chem. 237, 1657-1660

Barracclough, C.A. & Sawyer, C.H. (1955)

Inhibition of the release of pituitary ovulating hormone in the rat by morphine.

Endocrinology 57, 329-334

Barreclough, C.A. (1961)

Production of anovulatory, sterile rats by a single injection of testosterone propionate

Endocrinology 68, 62-67

Barracclough, C.A. & Wise, P.M. (1982)

The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion.

Endocr. Rev. 3, 91-119

- Barracclough, C.A., Wise, P.M. & Selmanoff, M.K. (1984)  
A role for hypothalamic catecholamines in the regulation of gonadotrophin secretion.  
Recent Prog. Horm. Res. 40, 487-529
- Baker, B.L., Dermody, W.C., & Reel, J.R. (1975)  
Distribution of gonadotrophin-releasing hormone immunocytochemistry  
Endocrinology 97, 125-135
- Ben-Jonathan, N., Oliver, C., Weiner, H.J., Mical, R.S. & Porter, J.C. (1977)  
Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy.  
Endocrinology 100, 452-458
- Benedetti, W.L., Sala, M.A. & Oregui, J.T. (1976)  
Persistent estrus in rats after anterolateral hypothalamic microinjections of 6-hydroxydopamine.  
Neuroendocrinology 21, 297-303
- Berson, S.A., Yalow, R.S., Bauman, W., Rothschild, M.A. & Newerly, K. (1956)  
Insulin-<sup>131</sup>I metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin-treated subjects.  
J. Clin. Invest. 35, 170-190
- Ben-Jonathan, N. & Porter, J.C. (1976)  
A sensitive radioenzymatic assay for dopamine, norepinephrine, and epinephrine in plasma and tissue.  
Endocrinology 98, 1497-1507

Blaschko, H.J. (1939)

The specific action of L-dopa-decarboxylase.

J. Physiol.(Lond.)96, 50-51

Blake, C.A. & Sawyer, C.H. (1974)

Effect of hypothalamic deafferentation on the pulsatile rhythm in plasma concentrations of luteinizing hormone in ovariectomized rats.

Endocrinology 94, 730-736

Blake, C.A. (1977)

A medial basal hypothalamic site of synergistic action of estrogen and progesterone on the inhibition of pituitary luteinizing hormone release.

Endocrinology 101, 1130-1134

Brownstein, M.J., Saavedra, J.M., Palovits, M. & Axelrod, J. (1974)

Histamine content of hypothalamic nuclei of the rat.

Brain Res.77, 151-156

Brownstein, M.J., Palkovits, M., Tappaz, M.L., Saavedra, J.M. &

Kizer, J.S. (1976)

Effect of surgical isolation of the hypothalamus on its neurotransmitter content.

Brain Res. 117, 287-295



Brown-Grant, K., Exley, D. & Naftolin, F. (1970)

Peripheral plasma oestradiol and luteinizing hormone concentrations during the oestrous cycle of the rat.

J. Endocr. 48, 295-296

Brown-Grant, K., Dutton, A., & ter Haar, M.B. (1977)

Variations in plasma thyrotrophin concentration during the rat oestrous cycle.

J. Endocr. 72, 33-34

Buckingham, J.C., Dohler, K.D. & Wilson, C.A. (1978)

Activity of the pituitary-adrenocortical system and thyroid gland during the oestrous cycle of the rat.

J. Endocr. 78, 359-366

Burnet, F.R. & Mackinnon, P.C.B. (1975)

Restoration by oestradiol benzoate of a neural and hormonal rhythm in the ovariectomized rat.

J. Endocr. 64, 27-35

Buttner, J., Borth, R., Boutwell, J.H., Broughton, P.M.G. & Bowyer, R.C. (1980)

Approved recommendation (1978) on quality control in clinical chemistry

J. Clin. Chem. Clin. Biochem. 18, 69-77

Caligaris, L., Astrada, J.J. & Taleisnik, S. (1971)

Release of luteinizing hormone induced by oestrogen injection into ovariectomized rats.

Endocrinology 88, 810-820

Caligaris, L., Astrada, J.J. & Taleisnik, S. (1972)

Influence of age on the release of luteinizing hormone induced by oestrogen and progesterone in immature rats.

J. Endocr. 55, 97-103

Caligaris, L., Astrada, J.J. & Taleisnik, S. (1978)

Release of luteinizing hormone induced by estrogen and progesterone in immature rats.

J. Endocr. 55, 93-103

Campbell, C.B.G. & Ramaley, J.A. (1974)

Retinohypothalamic projections: correlations with onset of the adrenal rhythm in infant rats.

Endocrinology 94, 1201-1204

Carmichael, S.W. & Winkler, Hans (1985)

The adrenal chromaffin cell.

Sci. Am. 253, 30-39

Channing, C.P., Schaerf, F.W., Anderson, L.D. & Tsafiriri, A. (1980)

Ovarian follicular and luteal physiology.

Int. Rev. Physiol. 22, 117-201

- Chappel, S.C., Ulloa-Aguirre, A. & Coutifairs, E. (1983)  
Biosynthesis and secretion of follicle-stimulating hormone.  
Endocr. Rev.4, 179-211
- Chalmers, J.P., Howe, P.R., Wallman, Y. & Tumulus, I. (1981)  
Adrenaline neurones and PNMT activity in the brain and spinal cord of  
genetically hypertensive rats and rats with doca-salt hypertension.  
Clinical Sci.(suppl)61, 2195-2215
- Ciaranello, R.D., Wong, D.L., Berenbeim, D.M. (1978)  
Regulation of pheylethanolamine-N-methyl transferase synthesis and  
degradation.  
Mol. Pharmacol.14, 490-501
- Ciaranello, R.D., Barchas, R.E., Byers, G.S., Stemmler, D.W. & Barchas,  
J.D. (1969)  
Enzymatic synthesis of adrenaline in mammalian brain.  
Nature (Lond.) 221, 368-369
- Cleland, (1964)  
Dithiothreitol, a new protective reagent for SH groups  
Biochem.3, 480-482
- Clayton, R.M. & Catt, K.J. (1981)  
Gonadotrophin-releasing hormone receptors: Characterization, physiological  
regulation and relationship to reproductive function.  
Endocr.Rev.2, 186-210

Clark, C.R., Maclusky, N.J. & Naftolin, F. (1982)

Oestrogen induction of progestin receptors in the rat brain and pituitary gland quantitative and kinetics aspects.

J. Endocr.93, 339-353

Clifton, D.K. & Sawyer, C.H. (1979)

LH release and ovulation in the rat following depletion of hypothalamic norepinephrine: Chronic vs acute effects.

Neuroendocrinology 28, 442-449

Coen, C.W. & Mackinnon, P.C.B. (1979)

Serotonin involvement in the control of phasic luteinizing hormone release in rat: evidence for a critical period.

J. Endocr.82, 105-113

Coen, C.W. & Mackinnon, P.C.B. (1981)

The effects of blocking catecholamine synthesis on the incidence of the LH surge and ovulation in the rat.

Neuroscience Lett.Suppl.7, S252

Coen, C.W., Coombs, M.C., Wilson, P.M.J., Clement, E.M. & Mackinnon, P.C.B. (1982)

Manipulating the LH surge and PCPA and 5HT: evidence for a mode of action involving adrenaline.

Neuroscience Suppl.7, S42

Coen, C.W., Coombs, M.C., Wilson, P.M.J., Clement, E.M., & Mackinnon, P.C.B. (1983)

Possible resolution of a paradox concerning the use of P-chlorophenyl-alanine and 5-hydroxytryptophan: Evidence for a mode of action involving adrenaline in manipulating the surge of luteinizing hormone in the rat.

Neuroscience 8, 583-591

Coyle, J.T. & Henry, D. (1973)

Catecholamines in fetal and newborn rat brain.

J. Neurochem.21, 61-67

Connet, R.J. & Kirshner, N. (1970)

Purification and properties of bovine phenylethanolamine-N-methyl transferase.

J. Biol.Chem.245, 329-334

Costa, E. & Neff, N.H. (1977)

Estimation of turnover rates to study the metabolic regulation of the steady state level of neuronal monoamines. In: Handbook of Neurochemistry, Lajtha, A. (Ed.), 45-90 Plenum Press New York.

Crowley, W.R. & Cass-Terry, L. (1981)

Effect of an epinephrine synthesis inhibitor SKF4139, on the secretion of luteinizing hormone in ovariectomized female rats.

Brain Res.204, 231-235

Crowley, W.R., Cass-Terry, L. & Johnson, M.D. (1982)

Evidence for the involvement of central epinephrine systems in the regulation of luteinizing hormone, prolactin and growth hormone release in female rats.

Endocrinology 110, 1102-1107

Cuello, A.C., Hiley, R. & Iverson, L.L. (1973)

Use of catechol-o-methyl transferase for the enzyme radiochemical assay of dopamine.

J. Neurochem.21, 1337-1340

Cuello, A.C. (1979)

The radioenzymatic assay of catecholamines. In: Neurobiology of dopamine, Korf, A.S., Westerick, B. & Korf, J. (Eds.), 77-88 Academic Press New York.

Cubeddu, X.L. & Vargas, A.M. (1977)

Effects of sodium chloride on phenylethanolamine-N-methyl transferase activity.

Mol. Pharmacol. 13, 172-180

Curtis, A. Lyons, V. & Fink, G. (1985)

The priming effect of LH-releasing hormone: effects of cold and involvement of new protein synthesis.

J. Endocr. 105, 163-168

Dahlstrom, A. & Fuxe, K. (1964)

Evidence for the existence of monoamine-containing neurons in the central nervous system I: Demonstration of monoamines in the cell bodies of brain stem neurons.

Acta Physiol. Scand. 62, 1-55

Day, T.A., Jervois, P.M., Manadue, M.F. & Willoughby, J.O. (1982)

Catecholamine mechanisms in the medio-basal hypothalamus influence prolactin but not growth hormone secretion.

Brain Res. 253, 213-219

Deguchi, T. & Barchas, J. (1970)

Inhibition of transmethylation of biogenic amines by S-adenosylhomocysteine.

J. Biol. Chem. 246, 3175-3181

DePrada, M. & Zurcher, G. (1976)

Simultaneous radioenzymatic determination of plasma and tissue adrenaline noradrenaline and dopamine with the femtomole range.

Life. Sci. 19, 1161-1174

DeLean, A., Garon, M., Kelly, P.A. & Labrie, F. (1977)

Changes of pituitary thyrotrophin releasing hormone (TRH) receptors level and prolactin response to TRH during the rat estrous cycle.

Endocrinology 100, 1505-1510

Dorrington, J.H., Moon, Y.S. & Armstrong, D.T. (1975)

Estradiol-17 $\beta$  biosynthesis in cultured granulosa cells from hypophysectomized immature rats: stimulation by follicle-stimulating hormone.

Endocrinology 97, 1328-1331

Dorrington, J.H. (1977)

Steroidogenesis in vitro In: Zuckerman L, Weir, B.J. (Eds) The Ovary, vol.3. Academic Press New York, 359-412

Döhler, K.D. & Wuttke, W. (1974)

Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats.

Endocrinology 94, 1003-1008

Döhler, K.D., Mühlen, A.V., Gärtner, K. and Döhler, U. (1976)

Various blood sampling techniques and the effects on pituitary hormones in rats.

Acta Endocr. 82 (suppl) 202, 29-30

Drouva, S.V., Laplante, E. & Kordon, C. (1982)

$\alpha_1$ -Adrenergic receptor involvement in the LH surge in ovariectomized oestrogen-primed rats.

European J. Pharmacol.81, 341-344

Drouin, J., Lagace, L. & Labrie, F. (1976)

Estradiol-induced increase of the LH responsiveness to LH releasing hormone (LHRH) in rat anterior pituitary.

Endocrinology 99, 1477-1481

Drouin, J. & Labrie, F. (1981)

Interactions between  $17\beta$ -estradiol and progesterone in control of LH and FSH release in rat anterior pituitary cells in culture.

Endocrinology 108, 52-57

Eldridge, J.C., Dmowski, W.P., Mahest, V.B. (1974)

Effect of castration of immature rats on serum FSH and LH and of various steroid treatments after castration.

Biol.Reprod.10, 438-446

Engelman, K., Portnoy, B. & Lovenberg, W. (1968)

A sensitive and specific double-isotopic derivative method for the determination of catecholamines in biological specimens.

Am.J.Med.Sci.255, 259-268

Engelman, K. & Portnoy, B. (1970)

A sensitive double-isotopic derivative assay for norepinephrine and epinephrine normal resting human plasma levels.

Circ.Res.26, 53-57



Erikson, B.M., Anderson, I., Borg, L.O. & Persson, B.A. (1977)

Determination of adrenaline and noradrenaline in plasma by an isotope derivative method and ion pair liquid chromatography

Acta Pharmacol. Suec. 14, 451-458

Everett, J.W., Sawyer, C.H. & Markee, J.E. (1949)

A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat.

Endocrinology 44, 234-250

Everett, J.W. & Sawyer, C.H. (1950)

A 24-hour periodicity in the "LH-release apparatus" of female rats, discovered by barbiturate sedation.

Endocrinology 47, 198-218

Everett, J.W. (1964)

Central control of reproductive functions of the adenohypophysis.

Physiol. Rev. 44, 373-431

Falck, B. (1959)

Site of production of oestrogen in rat ovary as studied in microtransplants

Acta Physiol. Scand. 47(Suppl.) 163, 1 - 101

Ferland, L., Marchetti, B., Segun, C., Lefebvre, F.A., Reeves, J.J. & Labrie, F. (1981)

Dissociated changes of pituitary LHRH receptors and responsiveness to the neurohormone induced by  $17\beta$ -estradiol and LHRH in vivo in the rat.

Endocrinology 109, 87-93

Ferin, M., Tempone, A., Zimmering, P.E., Vande Wiele, R.L. (1969)

Effect of antibodies to  $17\beta$ -estradiol and progesterone on the oestrous cycle of the rat.

Endocrinology 85, 1070-1978

Fink, G. (1976)

In ovulation in the human. Eds. P.G. Crosignani, D.R. Mishell, 95-114, Academic Press New York.

Fink, G. (1979)

Feedback actions of target hormones on hypothalamus and pituitary with special reference to gonadal steroids.

Ann.Rev.Physiol.41, 571-585

Fortune, J.E. & Armstrong, D.T. (1977)

Androgen production by theca and granulosa isolated from proestrous rat follicles.

Endocrinology 100, 1341-1347

Franks, W., Mason, H.D., Schenna, K.I.J. & Sheppard, M.C. (1984)

Stimulation of prolactin secretion by oestradiol in the rats is associated with increased hypothalamic release of thyrotrophin-releasing hormone.

J. Endocr.103, 257-261

Frantz, A.G., Kleinberg, D.L. & Noel, G.L. (1972)

Studies on prolactin in man.

Recent Prog. Horm.. Res.28, 527-590

Fuller, R.W. & Hunt, J.M. (1967)

Inhibition of phenylethanol-N-methyl transferase by its products epinephrine.

Life Sci.6, 1107-1112

- Fuller, R.W., Hemrick-Luecke, S., Toomey, R.E., Horng, J.S., Ruffolo, R.R. & Mollo, B.B. (1981)  
Properties of 8,9-dichloro-2,3,4,5-tetrahydro-1H-2-benzazepine, an inhibitor of norepinephrine N-methyl-transferase.  
Biochem.Pharmacol.30, 1345-1352
- Fuller, R.W. (1982)  
Pharmacology of brain epinephrine neurones.  
Ann.Rev.Pharmacol. Toxicol.22, 31-55
- Fuller, R.W. & Hemrick-Leucke, S.K. (1983)  
Evidence for inhibition of monoamine oxidase in vivo in rat brain by 7, 8-dichloro-1,2,3,4-tetrahydroisoquinoline.  
Res.Comm.Chem.Pathol.Pharmacol.39, 519-522
- Gauchy, C., Tassin, J.P., Glowinski, J. & Chéway, A. (1976)  
Isolation and radioenzymatic estimation of picogram quantities of dopamine and norepinephrine in biological samples.  
J. Neurochem. 26, 471-480
- Glowinski, J. & Iversen, L.L. (1966)  
Regional studies of catecholamines in the rat brain-I, The disposition of [ $^3\text{H}$ ] norepinephrine, [ $^3\text{H}$ ] dopamine and [ $^3\text{H}$ ] dopa in various regions of the brain.  
J. Neurochem. 13, 655-669
- Goodman, R.L. (1978)  
The site of the positive feedback action of estradiol in the rat.  
Endocrinology 102, 151-159

Gorski, R.A. (1963)

Modification of ovulatory mechanism by postnatal administration of  
oestrogen to the rat.

Am. J. Physiol. 205, 842-844

Goldman, B.D., Grazia, Y.R., Kamberi, I.A. & Porter, J.C. (1971)

Serum gonadotrophin concentrations in intact and castrated neonatal  
rats.

Endocrinology 88, 771-776

Green, J.D. & Harris, G.W. (1949)

Observations of the hypophysiportal vessels of the living rat.

J. Physiol. (London) 108, 359-361

Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963)

The preparation of <sup>131</sup>I-labelled human growth hormone of high specific  
activity.

Biochem. J. 89, 114-123

Gunne, L.M. (1962)

Relative adrenaline content in brain tissue.

Acta. Physiol. Scand. 56, 324-333

Gunne, L.M. (1963)

Catecholamines and 5-hydroxytryptamine in morphine tolerance and withdrawal.  
Acta. Physiol.Scand.58 (Suppl.), 1-91

Guldborg, H.C. & Marsden, C.A. (1975)

Catechol-o-methyl transferase pharmacological aspects and physiological  
role.

Pharmacol.Rev.27, 135-205

Guillemin, R. (1978)

Peptides in the brain: The new endocrinology of the neurone.

Science 202, 390-402

Hansson, E., Edhalm, L.E., Agrup, G., Rorsman, H., Rosengren, A.M. &

Rosengren, E. (1978)

The quantitative determination of 55-cysteinyldopa and dopac in man  
serum and in serum from patients with malignant melanoma by means of HPLC.

Clin.Chem.Acta.88, 419-427

Hanckle, J.L. & Wuttke, W. (1979)

Effects of chemical lesion of the ventral noradrenergic bundle of the  
medial preoptic area on preovulatory release in rats.

Exp. Brain Res.35, 127-134

Harms, P.G., Ojeda, S.R. & McCann, S.M. (1976)

Failure of monoaminergic and cholinergic receptor blockers to prevent  
prostaglandin E<sub>2</sub>-induced luteinizing hormone release.

Endocrinology 98, 318-323

Henderson, K.M. (1979)

Gonadotrophic regulation of ovarian activity.

British Medical Bulletin 35, 161-166

Hillier, S.G. (1981)

Regulation of follicular oestrogen biosynthesis: a survey of current concepts.

J. Endocr.89, 3P-18P

Hiemke, C., Bruder, D. & Ghraf, R. (1985)

Sex-specific effects of estradiol on hypothalamic noradrenaline turnover in gonadectomized rats.

Exp-Brain Res. 59, 68-72

Howe, P.R.C., Lovenberg, W. & Chalmers, J.P. (1981)

Increased number of PNMT-immunofluorescent nerve cell bodies in the medulla oblongata of a stroke-prone hypertensive rat

Brain Res.205, 123-130

Hökfelt, T. & Fuxe, K. (1972)

Brain endocrine interactions: On the morphology and the neuroendocrine role of hypothalamus catecholamine neurons. In: Median eminence structure and function, Knigge, K.M., Scott, W.W. & Weindle, A. (Eds), 181-223, Basel Karger.

Hökfelt, T., Fuxe, K., Goldstein, M. & Joh, T.H. (1973)

Evidence for adrenaline neurons in the rat brain.

Acta Physiol. Scand.89, 286-288

Hökfelt, T., Fuxe, K., Goldstein, M. & Johansson, O. (1974)

Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain.

Brain Res.66, 235-251

Hökfelt, T., Goldstein, M., Fuxe, K., Johansson, O., Verhofstad, A.,

Steinbusch, H., Penke, B. & Vargas, J. )1980)

Histochemical identification of adrenaline containing cells with special reference to neurones In: Central adrenaline neurones. Basic Aspects

and their role in cardiovascular Functions, Fuxe, K., Goldstein, M.,

Hökfelt, B. & Hokfelt, T. (Eds.), 19-47, Pergamon Press, Oxford/New York

Ieiri, T., Campbell, G.A. & Meites, J. (1980)

Effects of naloxone and morphine on proestrous surge of prolactin and gonadotropin in the rat.

Endocrinology 106, 1568-1578

Imai, K. & Tamura, Z. (1978)

Liquid chromatographic determination of urinary dopamine and norepinephrine as fluorscamine derivatives.

Clin.Chem.Acta 85, 1-6

Iversen, L.L. & Glowinski, J. (1966)

Regional studies of catecholamines in the rat brain - II, Rate of turnover of catecholamines in various brain regions.

J. Neurochem.13, 671-682

Johnston, C.A., Gibbs, D.M. & Negro-Vilar,A. (1983)

High concentrations of epinephrine derived from a central source and of 5-hydroxyindole-3-acetic acid in hypophysial portal plasma.

Endocrinology 113, 819-821

Kapeller, K. & Mayor, D. (1967)

The accumulation of noradrenaline in constricted sympathetic nerves as studied by fluorescence and electron microscopy.

Proc.R.Soc.B.167, 282-292

Kase, N.G. & Superoff, L. (1980)

The Ovary. In: Metabolic control and disease, Bondy, A.K. & Rosenberg, L.E. (Eds.), 319-374. Saunders & Co. Philadelphia

Kalra, P.S., Fawcett, C.P., Krulich, C. & McCann, S.M. (1973)

The effects of gonadal steroids on plasma gonadotropins and prolactin in the rat

Endocrinology 92, 1256-1268

Kalra, S.P. (1981)

Neural loci involved in naloxone-induced luteinizing hormone release: Effects of a norepinephrine synthesis inhibitor.

Endocrinology 109, 1805-1810

Kalra, S.P. & Simpkins, J.W. (1981)

Evidence for noradrenergic mediation of opioid effects on luteinizing hormone secretion.

Endocr.109, 776-782

Kalra, S.P., Simpkins, J.W. & Chen, C.L. (1981)

The opioid-catecholamine modulation of gonadotropin and prolactin secretion.

In: Functional correlates of hormone receptors in reproduction, Mahesh, V., Muldoon, T., Saxena, B. & Saddler, W. (Eds.), 135-150, Elsevier, Amsterdam.



Kalra, S.P. (1982)

Mode of opioid and catecholamine involvement in regulating LH secretion.

In: Hormonally active brain peptides, McKerns, K.W. & Pantic, V. (Eds.),  
Plenum Publishing Corporation.

Kalra, S.P. & Kalra, P.S. (1983)

Neural regulation of luteinizing hormone secretion in the rat.

Endocr.Rev.4, 311-351

Kawakami, M. & Terasawa, E. (1972)

Acute effect of neural deafferentation on timing of gonadotropin  
secretion before proestrus in the female rat.

Endocr. Japonica 19, 449-459

Kelly, M.J. (1982)

In electrical effects of steroids in neurons, McKerns, K.W. & Pantic.  
(Eds.), Hormonally active brain peptides, Structure and Function, Plenum  
Press New York

Kelly, M.J. & Ronnekleiv, O.K. (1982)

Evidence for estrogen responsive neurons that contain luteinizing hormone-  
releasing hormone in the medial basal hypothalamus of the guinea pig.

Endocrine Soc.64th Ann.Mtg.(Abstr), 615

King, J.C., Tobet, S.A., Snavely, F.L. & Arimura, A.A. (1982)

LHRH immunopositive cells and their projections to the median eminence  
and organum vasculosum of the lamina terminalis.

J. Comp. Neurol.209, 287-300

Kissinger, P.T., Riggin, R.M., Alcorn, R.L. & Leh-Daw, R. (1975)  
Estimation of catecholamines in urine by high pressure liquid chromatography with electrochemical detection.  
Biochem.Med.13, 299-306

Kirshner, N. & Goodall, M. (1957)  
The formation of adrenaline from noradrenaline.  
Biochem.Biophys.Acta 24, 658-659

Koslow, S.H. & Schlumpf, M. (1974)  
Quantitation of adrenaline in rat brain nuclei and areas by mass fragmentography.  
Nature (Lond.) 251, 530-531

Kordon, C., Kerdelh  , B., Pattou, E. & Justisz, M. (1974)  
Immunocytochemical localization of luteinizing hormone-releasing hormone in axons and nerve terminals of rat brain median eminence.  
Proc.Sc.Exp.Biol. & Med.147, 122-127

K  nig, J.F.R. & Klippel, R.A. (1963)  
The rat brain: A stereotaxic atlas of the forebrain and lower parts of the brain stem, Wilkins, Baltimore, Md.

Lew, J.Y., Matsumoto, Y., Pearson, J., Goldstein, M., H  kfelt, T. & Fuxe, K. (1977)  
Localization and characterization of PNMT in the brain of various mammalian species.  
Brain Res.119, 199-210

Legan, S.J., Coon, G.A. & Karsch, F.G. (1975)

Role of oestrogen as initiator of daily LH surges in the ovariectomized rat.

Endocrinology 96, 50-56

Legan, S.J. & Karsch, F.J. (1975)

A daily signal for the LH surge in the rat.

Endocrinology 96, 57-62

Lewis, C.E., Morris, J.F. & Fink, G. (1985)

The role of microfilaments in the priming effect of LH-releasing hormone: An ultrastructural study using cytochalasin B.

J. Endocr. 106- 211-218

Leong, D.A., Frawley, L.S. & Neill, J.D. (1983)

Neuroendocrine control of prolactin secretion.

Ann.Rev.Physiol.45, 109-127

Lichtensteiger, W. (1970)

Catecholamine containing neurones in neuroendocrine regulation: Principles and changes with microfluoremetry.

Prog. Histochem. Cytochem.1, 185-276

Lichtensteiger, W. & Keller, P.J. (1974)

Tubero-infundibular dopamine neurons and the secretion of luteinizing hormone and prolactin: Extrahypothalamic influences interaction with cholinergic systems and the effect of urethane anesthesia.

Brain Res.74, 279-303

Lostroh, A. & Johnson, R.E. (1966)

Amounts of interstitial-cell stimulating hormone and follicle-stimulating hormone required for development, uterine growth and ovulation in the hypophysectomized rat.

Endocrinology 79, 991-996

Löfström, A. (1977)

Catecholamine turnover alterations in discrete areas of the median eminence of the 4- and 5-day cyclic rat.

Brain Res. 120, 113-131

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951)

Protein measurement with the folin phenol reagent.

J. Biol. Chem. 193, 265-275

Loullis, C.C., Felten, D.L. & Shea, P.A. (1979)

HPLC determination of biogenic amines in discrete brain areas in food deprived rats.

Pharmacol. Biochem. Behaviour 11, 89-93

Luine, V.N. & McEwen, B.S. (1983)

Sex differences in cholinergic enzymes of diagonal band nuclei in the rat preoptic area.

Neuroendocrinology 36, 475-482

Luine, V.N., Khylicherskaya, R.I., McEwen, B.S. (1975)

Effect of gonadal steroids on activities of monoamine oxidase and choline acetylase in rat brain.

Brain Res. 86, 293-306

Mackinnon, P.C.B., Mattock, J.M. & ter Haar, M.B. (1976)

Serum gonadotrophin levels during development in male, female and androgenized female rats and the effect of general disturbance on high luteinizing hormone levels.

J. Endocr.70, 361-371

Mackinnon, P.C.B., Clement, E.M., Clark, C. & Sheaves, R. (1983)

Hypothalamic adrenergic activity precedes the preovulatory surge in the rat.

Neuroscience Lett.43, 221-226

Mackinnon, P.C.B., Clement, E.M. & Sheaves, R. (1985)

Changes in concentrations and rate of decline of adrenaline in the medial preoptic area and mediobasal hypothalamus of acutely ovariectomized steroid-treated rats.

J.Endocr.104,129-135

Martinovic, J.V. & McCann, S.M. (1977)

Effect of lesions in the ventral noradrenergic tract produced by microinjection of 6-hydroxydopamine on gonadotropin release in the rat.

Endocrinology 100, 1206-1213

Martini, L., Fraschini, F. & Motta, M. (1968a) In: Endocrinology and human behaviour, Michael, R.P. (Ed.), Oxford Univ. Press.

Martini, L., Fraschini, F. & Motta, M. (1968b)

Neural control of anterior pituitary functions.

Recent Prog.Horm.Res.24, 439-496

Marki, F., Axelrod, J. & Witkop, B. (1962)

Catecholamines and methyltransferase in the South America toad (*Bufo marinus*)

Biochem.Biophys.Acta 58, 367-369

Mackenzie, F.J., Hunter, A.J., Daly, C. & Wilson, C.A. (1984)

Evidence that the dopaminergic incerto-hypothalamic tract has a stimulatory effect on ovulation and gonadotrophin release.

Neuroendocrinology 39, 289-295

McCaman, R.E. (1965)

Microdetermination of catechol-o-methyl transferase in brain.

Life Sci.4, 2353-2359

McCaman, R.E., McCaman, M., Hunt, J.M. & Smith, S.M. (1965)

Microdetermination of monoamine oxidase and 5-hydroxytryptophan decarboxylase activities in nervous tissues.

J. Neurochem.12, 15-23

McCann, S.M. & Ramirez, V.D. (1964)

The neuroendocrine regulation of hypophysial luteinizing hormone secretion.

Recent Prog.Horm.Res.20, 131-181

McGeer, P.L. and McGeer, E.G. (1964)

Formation of adrenaline by brain tissue.

Biochem.Biophys.Res.Comm.17, 502-507

Meites, J., Bruni, J.F., Van Vugt, D.A. & Smith, A.F. (1979)

Relation of endogenous opioid peptides and morphine to neuroendocrine functions.

Life Sci.24, 1325-1336

Mell, L.D. & Gustafson, A.B. (1977)

Urinary free norepinephrine and dopamine determined by reverse-phase HPLC.

Clin.Chem.23, 473-476

Mefford, I., Oke, A., Adams, R.M. & Jonsson, G. (1977)

Epinephrine localization in human brain stem.

Neuroscience Lett.5, 141-145

Meijs-Roelofs, H.M.A., Uilenbroek, J.Th.J., De Greef, W.J., De Jong, F.H. & Kramer, P. (1975)

Gonadotrophin and steroid levels around the time of first ovulation in the rat.

J. Endocr.67, 275-282

Moore, C.L. & Price, D. (1932)

Gonadal hormone functions and the reciprocal influence between gonads and hypophysis with its bearing on sex hormone antagonism.

Am.J. Anat.50, 13-67

Moore, K.E. & Phillipson, O.T. (1975)

Effects of dexamethasone on phenylethanolamine N-methyl transferase and adrenaline in the brains and superior cervical ganglia of adult and neonatal rats.

J. Neurochem.25, 289-294

Moore, R.Y. & Bloom, F.E. (1979)

Central catecholamine neurone systems: Anatomy and physiology of norepinephrine and epinephrine systems.

Ann.Rev.Neurosci.2, 113-168

Moguilevsky, J.A., Faigón, M.R., Scacchi, P. & Szwarcfarb, B. (1981)

Hyperprolactinemia does not affect the development of the stimulatory mechanism of oestrogen-progesterone on LH secretion.

Acta Endocr.Copenh.98, 184-188

Moguilevsky, J.A., Faigon, M.R., Szwarcfarb, B., Seacchi, P. & Cardinali, D.P. (1982)

Factors influencing the positive feedback effect of ovarian steroids on LH secretion. In: Progress in clinical and biological research, Nicola, D.C., Blaquier, J. & Soto, R. (Eds.), 87, 49-59 Alan R. Liss, New York

Monroe, S.E., Parlow, A.F. & Midgley, A.R., Jr. (1968)

Radioimmunoassay for rat luteinizing hormone

Endocrinology 83, 1004-1012

Muth, E.A., Crowley, W.R. & Jacobowitz, D.M. (1980)

Effect of gonadal hormones on luteinizing hormone in plasma and on choline acetyl transferase activity and acetyl choline levels in discrete nuclei of the rat brain

Neuroendocr.30, 329-336



Naftolin, F., Corker, C.S. (1970)

An ultra-micro method for the measurement of luteinizing hormone by radioimmunoassay. In: Radioimmunoassay methods, Kirkham, K.E. & Hunter, W.M. (Eds.), 641-645, Churchill and Livingstone, London

Negro-Vilar, A., Ojeda, S.R. & McCann, S.M. (1980)

Hypothalamic control of LHRH and somatostatin: role of central neurotransmitters and intracellular messengers. In: Biochemical action of hormones, Litwack, C. (Ed.), Academic Press New York

Neill, J.D. (1974)

Prolactin: its secretion and control. In Handbook of physiology, Sect 7, Endocrinology, Knobil, E & Sawyer, W.H. (Eds.) 4, 469-488, Washington DC: Am. Physiol.Soc.

Nicholson, G., Greeley, G., Humm, J., Youngblood, W. & Kizer, J.S. (1978)

Lack of effect of noradrenergic denervation of the hypothalamus and medial preoptic area on the feedback regulation of gonadotrophin secretion and the estrous cycle of the rat.

Endocrinology 103, 559-566

Niswender, G.D., Midgley, A.R., Jr., Monroe, S.E. & Reichert, L.E., Jr. (1968)

Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine LH<sup>131</sup>I.

Proc.Soc.Exp.Biol. & Med. 128, 807-811

Nikodijevic, B., Daly, J. and Craveling, C.R. (1969)

Catechol-o-methyl transferase: An enzymatic assay for cardiac norepinephrine.

Biochem.Pharmacol.18, 1577-1584

Nunez, E.A., Benassayag, C., Savu, L., Valette, G. & Jayle, M.F. (1976)

Serum binding of some steroid hormone during development in different animal species. Discussion of the biological significance of this binding.

Ann.Biol.Anim.Biochem. Biophys.16, 491-505

Oliver, C., Mical, R.S., Porter, J.C. (1977)

Hypothalamic-pituitary vasculature-evidence for retrograde blood flow in the pituitary stalk.

Endocrinology 101, 598-604

Ojeda, S.R., Andrews, W.W., Advis, J.P. & Smith-White, S. (1971)

Recent advances in the endocrinology of puberty.

Endocr. Rev.1, 228-237

Ojeda, S.R., Wheaton, J.E. & McCann, S.M. (1975)

Prostaglandin E<sub>2</sub>-induced release of luteinizing hormone releasing factor (LRF)

Neuroendocrinology 17, 283-287

Ojeda, S.R., Negro-Vilar, A. & McCann, S.M. (1979)

Release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by hypothalamic tissue: evidence for their involvement in catecholamine-induced LHRH release

Endocrinology 104, 617-624

Ojeda, S.R., Negro-Vilar, A. & McCann, S.M. (1981)

Role of prostaglandins in the control of pituitary hormone secretion.

In: Physiopathology of endocrine diseases and mechanisms of hormone action, Soto, R.J., Denicola, A. & Blaquier, J. (Eds.), 229-247, Liss New York

Ojeda, S.R. & Negro-Vilar, A. (1984)

Release of prostaglandin  $E_2$  from the hypothalamus depends on extracellular  $Ca^{+2}$  availability : relation to LHRH release

Neuroendocrinology 39, 442-447

Ojeda, S.R. & Negro-Vilar, A. (1985)

Prostaglandin  $E_2$ -induced luteinizing hormone-releasing hormone release involves mobilization of intracellular  $Ca^{+2}$ .

Endocrinology 116, 1763-1770

Osland, R.B., Gallo, R.V. & Williams, J.A. (1975)

In vitro release of luteinizing hormone from anterior pituitary fragments superfused with constant or pulsatile amounts of luteinizing hormone releasing factor.

Endocrinology 96, 1210-1216

Palkovits, M. (1973)

Isolated removal of hypothalamic or other brain nuclei of the rat.

Brain Res. 59, 449-450

Palkovits, M., Brownstein, M., Saavedra, J.M. & Axelrod, J. (1974)

Norepinephrine and dopamine content of hypothalamic nuclei of the rat.

Brain Res. 77, 137-149

Palkovits, M. & Brownstein, M.J. (1983)

Microdissection of brain areas by the punch technique, In: Brain microdissection techniques, Cuellar, A.C. (Ed.), 1-36, John Wiley & Sons

Page, R.B., Munger, B.L. & Bergland, R.M. (1976)

Scanning microscope of pituitary vascular casts.

Am.J.Anat.146, 273-302

Passon, P. G. & Peuler, J.D. (1973)

A simplified radiometric assay for plasma norepinephrine and epinephrine.

Analytical Biochem.51, 618-631

Pang, C.N., Zimmerman, E. & Sawyer, C.H. (1977)

Morphine inhibition of the preovulatory surges of plasma luteinizing hormone and follicle stimulating hormone in the rat.

Endocrinology 101, 1726-1732

Pendleton, R.G., Khalsa, J., Gessner, G. & Sawyer, J. (1977)

Studies on the characterization and inhibition of rat brain phenylethanolamine-N-methyl transferase.

Arch.Pharmacol.299, 219-224

Peuler, J.D. & Johnson, G.A. (1977)

Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine.

Life Sci.21, 625-636

Pfaff, D.W. (1968)

Uptake of estradiol-17-<sup>3</sup>H in the female rat brain. An autoradiographic study.

Endocrinology 82, 1149-1155

Pfaff, D.W. & Keiner, M. (1973)

Atlas of estradiol concentrating cells in the central nervous system of the female rat.

J. Comp. Neurol. 151, 121-141

Pickering, A.J.M.C. & Fink, G. (1976)

Priming effect of LHRF - in vitro and in vivo evidence consistent with its dependence upon protein and RNA synthesis.

J. Endocr. 69, 373-379

Pickering, A.J.M.C. & Fink, G. (1979)

Priming effect of LHRF in vitro: role of protein synthesis, contractile elements,  $Ca^{++}$  and CAMP.

J. Endocr. 81, 223 - 224

Pohorecky, L.A., Zigmond, M., Karten, H. & Wurtman, R.J. (1969)

Enzymatic conversion of norepinephrine to epinephrine by the brain.

J. Pharmacol. Exp. Ther. 165, 190-195

Puig-Duran, E. & MacKinnon, P.C.B.M. (1978a)

A critical period for positive feedback in immature (21-day-old) rats.

J. Endocr. 76, 311-320

Puig-Duran, E. & MacKinnon, P.C.B.M. (1978b)

Ontogeny of prolactin and luteinizing hormone responses to oestrogen and progesterone in immature rats.

J. Endocr. 76, 321-331

Pycock, C.J. & Taberner, P.V. (Eds.) (1981)

Central neurotransmitter turnover, University Park Press Baltimore

Ramirez, V.D., Abrams, R.M. & McCann, S.M. (1964)

Effect of estradiol implants in the hypothalamo-hypophysial region of the rat on the secretion of luteinizing hormone.

Endocrinology 75, 243-248

Ramirez, V.D. & Sawyer, C.H. (1965)

Advancement of puberty in the female rat by estrogen.

Endocrinology 76, 1158-1168

Rance, N. & Barraclough, C.A. (1981)

Effects of phenobarbital on hypothalamic LHRH and catecholamine turnover rates in proestrous rats.

Proc.Soc.Exp.Biol.Med.166, 425-431

Rance, N., Wise, P.M., Selmanoff, M.K. & Barraclough, C.A. (1981)

Catecholamine turnover rates in discrete hypothalamic areas and associated changes in median eminence luteinizing hormone-releasing hormone and serum gonadotropins on proestrus and dioestrus day.

J. Endocr.108, 1795-1802

Raza-Bukhari, A., Parvez, S. & Parvez, H. (1976)

Influence of repeated injections of oestradiol during late pregnancy upon enzymes of monamine metabolism and biosynthesis in the rat.

Pharmacol.Res.Comm.8, 283-294

Raynaud, J.P., Mercier-Bodard, C. & Beaulieu, E.E. (1971)

Rat oestradiol binding plasma protein (EBP).

Steroids 18, 767-788

Refshauge, C., Kissinger, P.T., Dreiling, R., Blank, L.R., Freeman, R.  
& Adams, R.N. (1974)

New HPLC analysis of brain catecholamines.

Life Sci.14, 311-322

Read, G., Crabtree, B. & Smith, G.H. (1977)

The activities of 2-oxoglutarate dehydrogenase and pruvate dehydrogenase  
in hearts and mammary glands from ruminants and non-ruminants.

Biochem.J.164, 349-355

Richards, J.S. & Midgley, A.R.Jr.(1976)

Protein hormone action: A key to understanding ovarian follicular and  
luteal cell development.

Biol.Reprod.14, 82 - 94

Richards, J.S. (1978)

Hormonal control of follicular growth and maturation in mammals.

In: The vertebrate ovary, Ed.R.E. Jones, 331-360, New York Plenum Pres

Richards, J.S. (1979)

Hormonal control of ovarian follicular development: A 1978 perspective.

Recent Prog.Horm.Res.35, 343-373

Rubinstein, L. & Sawyer, C.H. (1970)

Role of catecholamines in the release of pituitary ovulating hormone(s)  
in rat.

Endocrinology 86, 988-995

Sarkar, D.K., Chiappa, S.A. & Fink, G. (1976)

Gonadotrophin-releasing hormone surge in pro-oestrous rats.

Nature 264, 461-463

Saper, C.B., Reis, D.J. & Joh, T.H. (1983)

Medullary catecholamine inputs to the anteroventral third ventricular cardiovascular regulatory region in the rat.

Neurosci Lett. 42, 285-291

Savoy-Moore, R.T., Schwartz, N.B., Duncan, J.A. & Marshall, J.C. (1980)

Pituitary gonadotrophin-releasing hormone receptors during the rat oestrous cycle.

Science 209, 942-944

Saavedra, J.M., Coyle, J.T. & Axelrod, J. (1973)

The distribution and properties of the non-specific N-methyl transferase in brain.

J. Neurochem. 20, 743-752

Saavedra, J.M., Palkovits, M., Brownstein, M.J. & Axelrod, J. (1974a)

Serotonin distribution in the nuclei of the rat hypothalamus and preoptic region.

Brain Res. 77, 157-165

Saavedra, J.M., Palkovits, M., & Axelrod, J. (1974b)

Localization of phenylethanolamine N-methyl transferase in the rat brain nuclei.

Nature, Lond. 248, 695-696



Saavedra, J.M. (1977)

Distribution of serotonin and synthesizing enzymes in discrete areas of the brain.

Fed.Proc.36, 2134-2141

Sar, M. & Stumpf, W.E. (1981)

Central noradrenergic neurons concentrate  $^3\text{H}$ -oestradiol.

Nature 289, 500-502

Sawyer, C.H., Markee, J.E. & Everett, J.W. (1950)

Further experiments on blocking pituitary activation in the rabbit and the rat.

J. Exp.Zool.113, 659-682

Saller, C.F. & Zigmond, M.J. (1978)

A radioenzymatic assay for catecholamines and dihydroxyphenyl acetic acid.

Life Sci.23, 1117-1130

Sauter, A.M., Baba, Y., Stone, E.A. & Goldstein, M. (1978)

Effect of stress and of phenylethanolamine-N-methyl transferase inhibition on central norepinephrine and epinephrine levels.

Brain Res. 144, 415-419

Sarkar, D.K., & Fink, G. (1980)

Luteinizing hormone releasing factor in pituitary stalk plasma from long-term ovariectomized rats: effects of steroids.

J. Endocr.86, 511-524

Schally, A.V. (1978)

Aspects of hypothalamic regulation of the pituitary gland.

Science 202, 18-28

Selmanoff, M.K., Pramik-Holdaway, M.J. & Weiner, R.I. (1976)

Concentration of dopamine and norepinephrine in discrete hypothalamic nuclei during the rat estrous cycle.

Endocrinology 99, 326-329

Seiden, G. & Brodish, A. (1972)

Persistence of a diurnal rhythm in hypothalamic corticotrophin-releasing factor (CRF) in the absence of hormone feedback.

Endocrinology 90, 1410-1403

Sherwood, N.M., Chiappa, S.A. & Fink, G. (1976)

Immunoreactive LHRH in pituitary stalk blood from female rats - sex steroid modulation of response to electrical stimulation of preoptic area or median eminence.

J. Endocr. 70, 501-511

Sherwood, N.M. & Fink, G. (1980)

Effect of ovariectomy and adrenalectomy on LHRH in pituitary stalk blood from female rats.

Endocrinology 106, 363-367

Sheaves, R., Warburton, E., Laynes, R. & Mackinnon, P. (1984)

Adrenaline concentration and turnover in the arcuate nucleus and median eminence during the critical period in the rat.

Brain Res. 323, 326-329

Sheaves, R., Laynes, R. & Mackinnon, P.C.B. (1985a)

Evidence that central epinephrine neurons participate in the control and regulation of neuroendocrine events during the estrous cycle.

Endocr. 116, 542-546

Sheaves, R., Laynes, R. & Mackinnon, P. (1985b)

Reduction of central epinephrine concentrations is consistent with the continued occurrence of ovulation in rats treated with an inhibitor (LY134046) of phenylethanolamine N-methyltransferase inhibitor (LY134046) of N-methyl transferase inhibitor (LY134046) of phenylethanolamine N-methyl transferase.

Neuroendocrinology 41, 432-436

Shivers, B.D., Harlan, R.E., Morrell, J.I. & Pfaff, D.W. (1983)

Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones.

Nature 304, 345-347

Speight, A. & Fink, G. (1981)

Changes in responsiveness of dispersed pituitary cells to luteinizing hormone releasing hormone at different times of the oestrous cycle of the rat.

J. Endocr. 89, 129-134

Stumpf, W.E. (1968)

Estradiol concentrating neurones: topography in the hypothalamus by dry mount autoradiography.

Science 162, 1001-1003

Stumpf, W.E. (1971)

Autoradiographic technique and the localization of estrogen, androgen and glucocorticoid in pituitary and brain.

Am.Zoologist 11, 725-739

Strauss, W.F. & Meyer, R.K. (1962)

Neural timing of ovulation in immature rats treated with gonadotrophin.

Science 137, 860-861

Swanson, L.W. and Hartman, B.K. (1975)

The central adrenergic system. An immunohistofluorescence study of the localization of cell bodies and their efferent connections in the rat utilizing dopamine- $\beta$ -hydroxylase as a marker.

J. Comp.Neurol.163, 467-506

Tapper, C.M., Greig, F. & Brown-Grant, K. (1974)

Effects of steroid hormones on gonadotrophin secretion in female rats after ovariectomy during the oestrous cycle.

J. Endocr.62, 511-525

ter Haar, M.B. & Wilson, C.A. (1978)

Evidence for pleiomorphism of luteinizing hormone in peripubertal female rats.

J. Endocr.79, 133-134

Toomey, R.E., Horng, J.S., Hemrick-Luecke, S.K. & Fuller, R.W. (1981)

$\alpha_2$ -adrenoceptor affinity of some inhibitors of norepinephrine N-methyl transferase.

Life Sci.29, 2467-2472

Turner, B.B., Timothy, E., Wilens, T.E., Schroeder, K.A., Katz, R.J.  
& Carroll, B.J. (1981)

Comparison of brainstem and adrenal circadian patterns of epinephrine  
synthesis.

Neuroendocrinology 32, 257-261

Ungar, A. & Phillips, J.H. (1983)

Regulation of the adrenal medulla.

Physiol.Rev.63, 787-843

Vijayan, E. & McCann, S.M. (1978)

The effects of systemic administration of dopamine and apomorphine on  
plasma LH and prolactin concentration in conscious rats.

Neuroendocrinology 25, 221 -235

Vogt, M.J. (1954)

The concentration of sympathin in different parts of the nervous system  
under normal conditions and after the administration of drugs.

J. Physiol. (Lond.) 123, 451-481

Waring, D.W. & Turgeon, J.L. (1980)

Luteinizing hormone-releasing hormone induced luteinizing hormone  
secretion in vitro: cyclic changes in responsiveness and self-priming.

Endocrinology 106, 1430-1436

Weiner, N. (1974)

A critical assessment of methods for the determination of monoamine  
synthesis turnover rates in vivo. In: Neuropsychopharmacology of  
monoamines, 143-159 Raven Press New York

Weiner, R.I. & Ganong, W.F. (1978)

Role of monoamines and histamine in regulation of anterior pituitary secretion.

Physiol.Rev.58, 905-976

Weise, V.K., & Kopin, I.J. (1976)

Assay of catecholamines in human plasma studies of a single isotope radioenzymatic procedure.

Life Sci.19, 1673-1686

Winer, B.J. (1971)

Statistical principles in experimental design., 2nd Edition Maytham, W., Shapiro, A and Stern, J. (Eds.) McGraw-Hill, New York

Wilson, C.A., ter Haar, M.B., Bonney, R.C., Buckingham, J., Dixon, A.F. & Yeo, T. (1983)

Hormonal changes in the immature rat after administration of pregnant mare serum gonadotrophin: influence of body weight.

J. Endocr.99, 63-76

Wilson, C.A., Buckingham, J.C. & Morris, I.D. (1985)

Influence of growth hormone, corticosterone, corticotrophin and changes in the environmental temperature on pituitary ovarian function in the immature rat.

J. Endocr.104, 179-183

Worthington, W.C. Jr. (1955)

Some observations on the hypophysial portal system in the living mouse.

Bull. John Hopkins Hosp.97, 343-357

Wurtman, R.J. & Axelrod, J. (1966)

Control of enzymatic synthesis of adrenaline in the adrenal medulla  
by adrenal cortical steroids.

J. Biol.Chem. 241, 2301-2305

Wurtman, R.J. (1966)

Control of epinephrine synthesis in the adrenal medulla by the adrenal  
cortex: Hormonal specificity and dose response characteristics.

Endocrinology 79, 608-614

Yalow, R.S. & Berson, S.A. (1960)

Immunoassay of endogenous plasma insulin in man.

J. Clin. Invest.39, 1157-1175

Yu, P.H. (1978)

Phenylethanolamine-N-methyl transferase from the brain and adrenal  
medulla of the rat.

Neurochem.Res.3, 755-762

Zammit, V.A. & Newsholme, E.A. (1976)

The maximum activities of hexokinase, phosphorylase, phosphofructokinase,  
glycerol phosphate dehydrogenase, lactate dehydrogenase, octopine  
dehydrogenase, phosphoenolpyruvate oxaloacetate transaminase and arginine  
utilization in muscles from marine invertebrates.

Biochem.J.160, 447-462

## APPENDIX

### PROTEIN ESTIMATION

The NaOH hydrolysate obtained by dissolving the pellet in 2M NaOH [see sections on radioenzymatic assay of catecholamines and phenylethanolamine-N-methyl transferase assay] was taken for estimation of protein content based on the method of Lowry et al. (1951).

#### 1. Reagents

(a) Bovine serum albumin (BSA) fraction V [Sigma Chemical Co., U.K.] stock solution: 100mg BSA was weighed in a silanized glass scintillation vial and added to it 10ml of 2M NaOH. It was left for gentle stirring until dissolved completely and then kept at 4°C until used. This solution can be used for at least one month (the maximum storage time used) if kept at 4°C.

#### (b) Standards:

µg BSA in 50µl sample	Standard soln.
0	2M NaOH
5	9.9ml 2M NaOH + 100µl Stock BSA
10	9.8ml 2M NaOH + 200µl Stock BSA
15	9.7ml 2M NaOH + 300µl Stock BSA

#### (c) Solution 1:

- (a) 10ml 2% (w/v)  $\text{Na}_2\text{CO}_3$
- (b) 0.1ml 1% (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- (c) 0.1ml 1% (w.v) NaKTartrate

The above three were kept as stock solutions at 4°C and mixed on the day of assay in this ratio (10:0.1:0.1).

#### (d) Solution 2:

50% (v/v) Folin Ciocalteu (F & C) reagent.



$\text{Na}_2\text{CO}_3$ ,  $\text{CuSO}_4$  and NakTartrate were obtained from Sigma Chemical Co., U.K. and F & C reagent was obtained from B.D.H. Ltd., U.K. Concentrated solution of F & C reagent and the solutions of  $\text{Na}_2\text{CO}_3$ ,  $\text{CuSO}_4$  and NakTartrate keep very well for months if kept at 4°C.

Solutions 1 and 2 were made up fresh for each experient.

## 2. Standard Assay Procedure

To ensure that proteins are digested completely, the LP2 capped tubes containing the hydrolysate were heated at 60°C for one hour. 2 x 50 $\mu$ l of the soluble hydrolysate were added by Hamilton syringe to glass tubes [2" x <sup>3</sup>/<sub>8</sub>" ] containing 1ml of 'solution 1'. Ten minutes later, 0.1ml 'solution 2' was added followed immediately by vortex-mixing briefly. The absorbance was measured against reagent blank at 750nm after one hour using Perkin-Elmer spectrophotometer and BSA standards were run in parallel.

A typical standard protein curve is shown in Fig.A. and the unknown samples were read off this curve. A range of unknown tissue samples which were read off the curve is shown by the hatched area. Duplicate measurements in the same assay that deviated by more than 10% from their mean value were discarded and the assay repeated.

## 3. Calculations

Although some authors have reported the determination of protein content present in the homogenate (Brownstein et al., 1974; Palkovits et al., 1974), Loullis et al. (1979) made use of the pellets for the determination of protein content. Since 2 x 10 $\mu$ l of the actual supernatant is an essential requirement [see Section 'C' of Chapter 2] for the determination of catecholamines and adrenaline concentration is much lower than dopamine and noradrenaline, it was not feasible to use the homogenate for protein determination. The protein content was therefore

measured in the pellets after digestion in 2M NaOH, accordance with Loullis et al. (1979). A preliminary study showed that 60% of protein appeared in the pellet and the remainder in the supernatant. Correction for missing 40% was made in order to get the total protein. Validity of this procedure was indicated by its consistency in protein content of the pellets from sample to sample. The determination of the total protein content was routinely performed by using a pre-programmed computer [Apple II].

### Precision

The precision is a measurement of coefficient of variation as discussed in section 'A' of this chapter. Intra - and inter - assay coefficients of variation were determined by using 8 aliquots of the same sample within the same assay and in separate assays respectively.

Intra-assay coefficient of variation:

$$\text{C.V. (\%)} = \frac{\text{S.D.}}{\text{Mean}} \times 100$$

$$\text{C.V.} = 4.22\%$$

Inter-assay coefficient of variation:

$$\text{C.V.} = 6.20\%$$

### 5. Sensitivity and Specificity

On the basis of twice the standard deviation (2 x S.D.) of blanks, the sensitivity resulted in 0.99 µg. However, the unknown samples never approached this limit since the estimated protein content ranged from 3µg - 7µg [shown by hatched area in Fig.A].

Specificity is determined at two steps which lead to the final colour

(i) protein reaction with the alkaline copper to form protein-copper complex,

(ii) reduction of phosphomolybdate with protein-copper complex to give blue colour.

Fig.A. Relationship of protein (BSA) content to absorbance (750nm) as determined by the Lowry-protein assay.

The protein range (3 - 7 $\mu$ g) in the NaOH extract of the standard assay for brain punches is shown by the hatched area.

Each point is a mean of 3 determinations.

